RABIES VIRUS SOLUBLE ANTIGENS.

A Biochemical and biophysical study of the major antigen of Rabies Virus.

by

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CHAPTER ONE

SUMMARY

The purpose of these investigations was to isolate and purify the largest of several antigens demonstrable in rabies-infected suckling mouse brains. The biochemical and biophysical properties of the antigen were studied with a view to elucidating its contribution to the intracellular synthesis and the structure of the virus particles.

Extracts of normal and infected suckling mouse brains were purified by precipitation at pH 4.5 and freed of the smaller antigens by centrifugation prior to digestion with RNAase, DNAase and trypsin. The large antigen was purified by enzyme treatment, preparative ultracentrifugation, exclusion chromatography and gradient centrifugation and appeared as rings varying in diameter between 8 and 12 mμ when examined by electron microscopy.

Methods for the chemical estimation of pentose, deoxypentose and nitrogen were modified to meet the requirements of this investigation, and these techniques were used to determine the composition of the purified antigen. The antigen is a ribonucleoprotein, and found to be resistant to RNAase, DNAase, trypsin and chymotrypsin.

A purified solution of the antigen contained 7.3 µg RNA/ml., 11.4 µg protein/ml. and probably a trace of DNA.

The success of this programme has resulted in the accumulation of certain original information which has been used in clarifying the nature and structure of the largest soluble antigen.

CHAPTER TWO

INTRODUCTION

Soluble antigens of rabies virus were discovered by Polson and Wessels (1953) who showed that extracts of rabies-infected suckling mouse brains contained, in addition to the virus, one or more soluble antigens which could be detected by complement fixation. After extraction and partial purification the soluble antigen appeared to have a diameter of 12.4mμ as determined by diffusion. On centrifugation a sedimentation coefficient of 25S was obtained.

Van den Ende, Polson and Turner (1957) homogenized rabies-infected brain material, freed the homogenate of virus and gross particulate matter by centrifugation and dialysed the supernatant fluid against citrate buffer pH 4.3. The precipitate formed on dialysis was extracted with alkaline phosphate buffer, and the supernatant retained as a source of soluble antigen.

In addition they found that on ultrasonicating a virus suspension freed of soluble antigen, or by alternate freezing and thawing of this suspension, such small amounts of soluble antigen were released that they were not considered to be constituents of the virus particle released on its disruption. The complement-fixing activity of the virus-

free extract was found to be partially resistant to digestion with trypsin and to be stable between pH 6 and 10.

In an attempt to purify the soluble antigen from infected brain material, Mead (1962a) undertook a complete re-investigation of the problem. The soluble antigens were partially purified by acid precipitation and alkaline extraction. Other methods tested included solvent treatment with chloroform, acetone and difluorotetrachloroethane, adsorption on calcium phosphate (Hydroxyapatite) and chromatography on diethylaminoethyl cellulose (DEAE), carboxymethyl cellulose (CM) and triethylaminoethyl cellulose (TEAE). Electrophoresis in starch gels, paper, glass fibre, cellulose acetate strips and starch grains was also investigated.

The most effective initial step in the purification was the acid precipitation at pH 4.5 followed by the extraction of the precipitate with alkaline buffer which was shown to eliminate 90% of the initial protein (including haemoglobin), but up to 70% of the complement-fixing activity was also lost. Batch adsorption on calcium phosphate and DEAE cellulose waspartly effective. However electrophoresis of crude and acid precipitated purified material was not very successful in purifying the antigens. The considerable losses of complement-fixing activity accompanying all the procedures that were tried suggested that

the antigen or antigens were unstable. Efforts to overcome this were unsuccessful, but freeze-dried material appeared to retain its complement fixing activity unimpaired.

In a subsequent investigation, Mead (1962b) showed that concentrated virus-free extracts gave four lines of precipitation in the Ouchterlony test with concentrated mouse antirabies serum as source of antibody. Extracts partially purified by acid precipitation and other methods, gave two or occasionally three lines and evidence was presented indicating that the crude mixture contained at least three antigens. The large or outer antigen sedimented slowly at 70,000g and was resistant to trypsin digestion. The three smaller antigens found in conjunction with the larger component were all sensitive to trypsin. Brain impurities which closely resembled the antigens in electrophoretic mobility, chromatographic behaviour and rate of sedimentation tended to make the purification of the antigen difficult.

Following upon the investigations of Mead, (1962 a, b,) the large antigen was chosen for investigation because it could be separated from the smaller antigens by centrifugation and because it was resistant to digestion with trypsin. The purification and

biochemical properties of the largest soluble antigen isolated from rabies-infected suckling mouse brains were investigated in these laboratories under the supervision of and in collaboration with Dr. T.H. Mead.

Most of the work undertaken at present throughout the world is concerned with the production of a safe and effective vaccine against rabies infection. Consequently investigations concerning the virus particle and especially the virus antigens, found in conjunction with the latter particle during multiplication in the host cell, have not been pursued. The largest rabies soluble antigen was investigated in this laboratory by Katz, Larsson and Mead (1967). The work described in this thesis deals briefly with the extraction, purification and biochemical properties of this antigen. Emphasis has, however, been placed on the modification of the biochemical techniques used for estimating RNA, DNA and nitrogen in the purified sample of the largest rabies specific soluble antigen.

Furthermore, it was hoped that the biochemical properties and the configuration and size of the antigen obtained from electron micrographs would be of value in assessing the role of the particle during virus multiplication in the cell.

CHAPTER THREE

MATERIALS AND METHODS

Introduction. The materials and general methods described in this chapter were used throughout this investigation; and more specialized techniques are described at the beginning of the succeeding chapters.

Chemicals and Buffers. Chemicals were of analytical quality, and the water used in the preparation of buffers was condensed in an all-glass apparatus.

Buffers.

Buffered saline (BS) pH 7.0. Mead (1962 a).

NaCl	17.0g.
0.2M-Na_2HPO_4	68 ml.
Dist. water.	1,800 ml.
Thiomersalate	0.2g.

adjust to pH 7.0 with 0.2M-KH_2PO_4 and dilute to 2 litres with water.

Buffered saline A. (Katz, Larsson and Mead 1967).

NaCl	85g.
Dist. water.	9000 ml.
0.2M-Na_2HPO_4	327 ml.
Triton X-100 (Rohm and Haas Co.)	1g.
Thiomersalate	1g.

adjust to pH 7.5 with 0.2M-KH_2PO_4 and dilute to 10 litres with distilled water. Triton X-100 was added to reduce protein denaturation at the surface of the liquid.

Tris-saline pH 8.4. (Katz, Larsson and Mead 1967).

2-amino-2-hydroxymethylpropane-1:3 diol.	2.4g.
NaCl	2.34g.
Triton X-100.	0.04g.
Thiomersalate.	0.04g.
Dist. water.	400 ml.

adjust to pH 8.4 with 1 N-HCl.

Acid saline pH 4.5. (Katz, Larsson and Mead 1967).

NaCl	8.5g.
Dist. water.	900 ml.
Glacial acetic acid.	0.6 ml.

adjust to pH 4.5 with 2 N-NaOH and dilute to 1 litre with distilled water.

EDTA saline pH 7.0. (Mead 1962a).

NaCl	17.0g.
Disodium salt of diaminoethanetetra-acetic acid (EDTA).	7.4g.
Dist.water.	1,500 ml.
0.2M-Na_2HPO_4	63 ml.
Triton X-100.	0.2g.
Thiomersalate.	0.2g.

adjust to pH 7.0 with 2 N-NaOH and dilute to 2 litres with distilled water.

Veronal buffered saline (CFT saline) recommended by Mayer, Osler, Bier and Heidelberger, (1946) was used as a diluent for complement fixation titration.

Solution 1.

NaCl	85g.
Sodium diethylbarbiturate	3.75g.
Dist. water.	1,400 ml.

Solution 2.

Hot dist. water ($\pm 70^{\circ}$).	500 ml.
Diethylbarbituric acid.	5.75g.

The second solution was added to the first and the following salts added after the above-mentioned mixture had cooled.

$MgCl_2 . 6H_2O$	1.68g.
$CaCl_2$	0.28g.

The solution was transferred to 1 litre screw cap bottles and autoclaved at 15 lbs/sq.in. for 30 min.

0.05M-Tris pH 8.6.

2-amino-2-hydroxymethylpropane-1:3 diol.	6.05g.
Dist. water.	500 ml.

adjust to pH 8.6 with 0.1 N-HCl and dilute to 1 litre with water.

Thiomersalate saline. (Mead 1962a).

NaCl	8.5g.
Thiomersalate.	0.1g.

Dilute with distilled water to 1 litre.

0.1M-Tris pH 7.6.

2-amino-2-hydroxymethylpropane-1:3 diol.	12.1g.
Dist. water.	900 ml.

adjust to pH 7.6 with 1 N-HCl and dilute to 1 litre with water.

Nigrosine stain. (Kohn, 1958)

Nigrosine	0.002g.
Acetic acid (2% $^{v}/_{v}$).	1000 ml.

Virus. The egg-adapted Flury strain of rabies virus as described by Van den Ende, Polson and Turner (1957) was used in the present work. Infective material from the seventeenth passage in suckling mouse brains was freeze-dried and working stocks were prepared at intervals from the latter material as follows. Three to four day-old suckling mice were inoculated intracerebrally with 0.02 ml. of a virus suspension prepared by resuspending the freeze-dried material in buffered saline containing 5% ($^{v}/_{v}$) normal mouse serum. The mice were dead or moribund on the third or fourth day after inoculation, and the brains were harvested immediately and temporarily stored at -20°. When required this was thawed and ground with sterile glass and buffered saline in a mortar and the emulsion centrifuged at 2000 rev./min. for 15 min.; the supernatant fluid was freeze-dried in ampoules and stored at 4°.

Mouse anti-rabies sera were prepared for use in complement fixation titrations and as a source of antibody in gel precipitin tests. This serum was concentrated for use in gel precipitin tests by freeze-drying and redissolving it in water to one third of its original volume.

Approximately 200 adult mice received 10 - 13 intraperitoneal injections (0.2 ml.) of a 10% ($^{w}/_{v}$) suspension of freshly prepared infected suckling mouse brains

(see above) at 3 - 4 day intervals. Only the first dose was inactivated with formalin (0.35%). One week after the final inoculation the animals were killed in ether vapour, the thoracic cavity opened and blood collected from the heart. The blood was kept at room temperature ($\pm$ 25°) for 1 hr. and then at 4° for approximately 14 hr. The serum was removed and inactivated at 56° for 30 min. and centrifuged at 59,000g for 1 hr. under liquid paraffin to remove lipids (Mead, 1962 a). The centrifuge tube was pierced at the bottom and 3/4 of the serum was collected from each tube, treated with thiomersalate (1/10,000 $^{w}/_{v}$) and stored at 4°.

Mouse Antinormal mouse brain serum was prepared by inoculation of adult mice with normal healthy suckling mouse brain suspensions as described above. This serum was used as a negative control in gel precipitin tests and was concentrated as described above (Mead, unpublished).

Normal mouse serum obtained from normal healthy adult mice was prepared as described above but was not certrifuged. This serum was used as a negative control in gel precipitin tests, and as a preservative in the virus diluent.

Jasus lalandii haemocyanin. This protein was used as a reference standard for exclusion chromatography and gradient centrifugation experiments. The protein was obtained from

the blood of male rock lobsters (Jasus lalandii) by severing the arteries on the ventral surface between the first segment of the abdomen and the carapace. After clotting, the blood was transferred to a muslin bag and gently squeezed to express the serum which was then clarified for 20 min. by centrifugation at 10,000 rev./min. The serum was not freeze-dried as its stability under these conditions was not known, but it was found necessary to add thiomersalate as a preservative because in its absence the serum developed a brownish discolouration which was an indication that it had broken down, even though it had been kept at 4°. Consequently thiomersalate (1/10,000 $^{w}/_{v}$) was added immediately after preparation of the serum.

Sonication. An MSE Mullard ultrasonic generator with a probe uniformly 3/4in. diameter and a power rating of 60 watts at 20 Kcyc./sec. was used. Homogenized brain suspensions were ultrasonicated in 25 ml. portions for 5 min. in a thick walled glass test tube. The probe was extended to a depth of approximately 1 mm. below the surface of the suspension which was surrounded by a mixture of crushed ice and water during the process.

Comminution of the brain material was carried out in a stainless steel "top drive" blender at 14,000 rev./min. (Mead 1962a).

Complement fixation titrations (CFT) were performed according to the method of Casals and Olitsky (1950) with modifications as described by Mead (1962a). This was at first the only method available for detecting the soluble antigen and remained the only method for estimating the quantitative amounts present in solutions suspected of containing it.

Preparation of the standard for CFT was carried out as follows. Approximately 20-30 litters of 3-4 day old suckling mice were inoculated intracerebrally with 0.02 ml. of a suspension of infected brains. When the mice were sick the brains were harvested and homogenized in 75g. distilled-water ice and 225 ml. cold BS pH 7.0. The emulsion was ultrasonicated and centrifuged at 44,000g in the No. 30 Spinco rotor for 2 hr. The supernatant fluid was transferred to a cellulose dialysis bag and "perfrigerated" overnight at 4°. The technique of perfrigeration was described by Mead (1962 a) and was used for concentrating solutions. The dialysis sac containing the liquid to be concentrated was placed immediately beneath the cooling element of the refrigerator. A small 12 volt direct current electric fan was placed below the sac to accelerate the process of evaporation by circulating the air in the refrigerator. Any water vapour in the

atmosphere of the refrigerator was collected as ice on the coils.

When the volume had been reduced sufficiently the sac was removed and the concentrated brain extract dialysed against 5 litres of water containing thiomersalate (1/10,000 $^{w}/_{v}$) for approximately 14 hr. at 4°. This material, which constituted the standard, was freeze-dried and stored at 4°.

Complement. Pooled guinea-pig serum was used as a source of complement and was preserved according to the method of Richardson (1941).

Solution A	Boric acid (H_3BO_3)	1.55 g.
	Saturated NaCl in dist. water to	100 ml.
Solution B	Sorbitol ($C_6H_{14}O_6.\frac{1}{2}H_2O$).	9.55 g.
	Sodium azide (NaN_3).	0.81 g.
	Saturated NaCl in dist. water to	100 ml.
Solution C	Sodium azide	0.81 g.
	Saturated NaCl in dist. water to	100 ml.

To 8 ml. of guinea-pig serum was added 1 ml. of solution A and 0.5 ml. of solutions B and C. The preserved complement was stored at 4°.

Sheep red cells. Blood was collected aseptically from the jugular vein of a sheep into 100 ml. 3.8% ($^{w}/_{v}$) sodium citrate in saline and 50 ml. Alsever's solution, which was prepared according to the method of Bukantz, Rein and Kent (1946).

Alsever's solution.

Dextrose	20.5 g.
Sodium citrate	8.0 g.
Sodium chloride	4.2 g.
Citric acid (to adjust pH to 6.1)	0.55 g.
Distilled water to	1000 ml.

The cells were left in this solution at 4° for approximately one week prior to their use. The erythrocytes in a sample of blood were washed four times with 4 - 5 volumes of BS pH 7.0 by alternate light centrifugation and resuspension of the cells. After the final centrifugation the supernatant was removed and the cells resuspended in 2 - 3 ml. of buffer.

The haemoglobin concentration of the cell suspension was determined with a Hemometer (Adams, U.S.A.) as follows. The cells were well mixed and 0.02 ml. pipetted into a graduated (grams % haemoglobin) test tube containing $\pm$ 1 ml. N/10 HCl. The cells and acid were well mixed producing a brown solution on lysis of the cells, this colour being matched against a standard by the addition of a few drops of acid until the colour intensities were the same. The concentration was read directly from the graduated tube and the remainder of the washed cells diluted with CFT saline to give a 3g %

concentration of haemoglobin for use in CF titrations. The cells were stored at 4° for no longer than 2 days prior to their use.

Haemolysin, was obtained from the stocks regularly used in the department of Bacteriology, Medical School, Cape Town. It was prepared according to the method of Kolmer, Spandling and Robinson (1951) and had a titre of 1/8000. The haemolysin was diluted 1/1,000 with CFT saline to give 8 minimal haemolytic doses (MHD) and stored at 4°.

Sheep cells were sensitized at room temperature (± 25°) by adding an equal volume of dilute haemolysin (8 MHD) to the 3% cell suspension. The mixture was stirred frequently during the 15 min. sensitization period and was used immediately after.

Titration of complement. The preserved complement was diluted in CFT saline (1/30 or 1/40) and then diluted further asshown in Table 1.

Table No. 1

Dilutions of complement with CFT saline

	Volumes in ml.										
Complement	2.0	1.6	1.4	1.2	1.0	0.9	0.8	0.7	0.6	0.5	0.4
CFT saline	1.0	1.4	1.6	1.8	2.0	2.1	2.2	2.3	2.4	2.5	2.6

To 0.3 ml.of each of the above dilutions was added 0.1 ml. of C.F.T. saline and 0.2 ml. sensitized cells. After having been shaken vigorously, the tubes were placed in a water bath at 37° for 30 min. The highest dilution showing complete haemolysis contained one minimal haemolytic dose. Two MHD's were used in CF titrations.

Titration of the mouse antirabies serum was done to determine the optimal concentration for use in CF titrations. Serum which was too concentrated was anticomplementary, and if used too dilute the test failed to reach an end point. The antirabies serum was diluted with CFT saline to give the following concentrations; 1/10, 1/20, 1/30, 1/40 and 1/50. A standard of known titre was diluted logarithmically covering a range of one log in ten steps (Mead 1962a) as shown in Table 2.

Table No. 2.

Logarithmic dilutions of standards and antigens.

	Volumes in ml.										
Standard or antigen	1.0	0.8	0.63	0.5	0.4	0.32	0.25	0.2	0.16	0.12	0.1
CFT saline	0	0.2	0.37	0.5	0.6	0.68	0.75	0.8	0.84	0.88	0.9

Five rows, each containing eleven tubes, were placed in a rack. Each row of tubes contained 0.1 ml. of each standard dilution. To the first row of tubes was added 0.1 ml. of

1/10 diluted antirabies serum, and to the second row was added 0.1 ml. of 1/20 diluted serum and so on until the final row had been completed. The tubes were kept at 4^{o} overnight, and the following morning, after having warmed to room temperature, a further 0.2 ml. of sensitized cells was added to each tube. After immersion in a water-bath at 37^{o} for 30 min. the tubes were read. The tube showing 50% haemolysis was taken as the end point of the titration. Serum diluted 1/20 or 1/30 was found to give the most satisfactory results.

Complement fixation titrations were performed on samples suspected of containing soluble antigen. Logarithmic dilutions of a standard and of solutions under investigation were prepared for each titration as shown in Table 2.

Two rows, each containing eleven tubes, were set up for the standard and for each solution suspected of containing soluble antigen. To each of the two rows was added 0.1 ml. of each of the dilutions of the standard or antigen. The first row contained a further 0.1 ml. of dilute antirabies serum, and the second 0.1 ml. CFT saline (control tubes). All the tubes received 0.2 ml. dilute complement.

Concurrent with the above titration, a serum control was prepared which contained 0.1 ml. CFT saline, 0.1 ml. serum and 0.2 ml. complement. The cell control

contained 0.4 ml. diluent. The complement control tubes contained respectively; (a) 0.05 ml. complement and 0.35 ml. diluent, (b) 0.1 ml. complement and 0.3 ml. diluent, (c) 0.2 ml. complement and 0.2 ml. diluent and (d) 0.4 ml. complement. After the addition of complement all the tubes were shaken and kept at 4° overnight. The following day the tubes were brought to room temperature prior to the addition of 0.2 ml. sensitized cells. The tubes were immersed in a water-bath at 37° for 30 min. and then read. The end point of the titration which was determined by eye, was determined on an empirical scale. A 100% haemolysis was read as 0 and no haemolysis as 4, the end point was taken as 2 which was equivalent to 50% haemolysis.

Agarose. This material was used in gel precipitin tests and in the preparation of pearls for exclusion chromatography. Araki (1958) showed that agar consists of two components: a charged sulphated polysaccharide, agaropectin, and a neutral galactose polymer agarose.

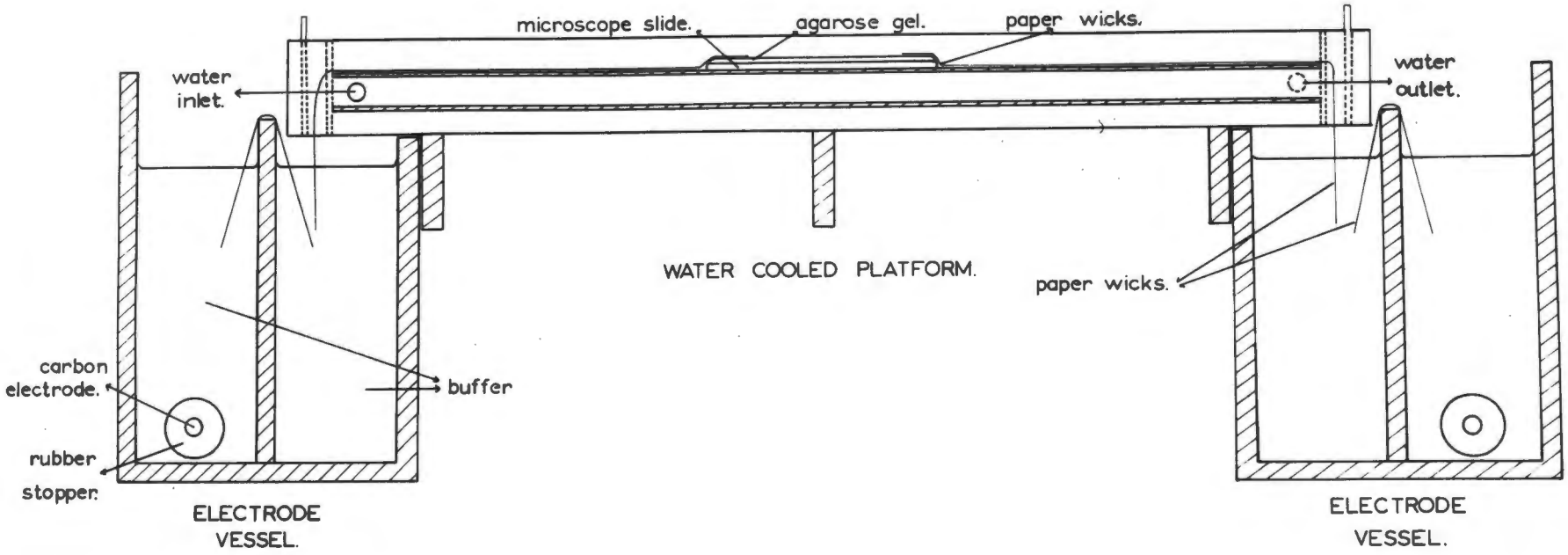
A number of methods exist for the separation of these two components, but that described by Russell, Mead and Polson (1964) was found to be the most effective and was used for preparing agarose as follows.

One hundred grams of Ionagar No 2 (Oxo Ltd., London) were dissolved in 2.5litres distilled water over a

boiling water-bath, with stirring. An equal volume of a 40% ($^{w}/_{v}$) solution of polyethylene glycol (PEG mol. wt. 6000) was added to the agar solution, both at 80°. The agarose precipitated almost immediately and was separated from the mother liquor, which contained most of the charged agaropectin in solution, by filtering through a nylon cloth of 110 mesh. The agarose was washed with distilled water until free of PEG, and then dehydrated by shaking in acetone, and finally by drying in a current of warm air. Three precipitations yielded agarose of a good quality. The amount of electroendosmotic flow produced when an electric current was passed through the gel was used as a measure of the purity of the agarose. The electrophoretic test was done as follows. A 1% ($^{w}/_{v}$) solution of agarose was prepared in 0.05M tris buffer pH 8.6 by heating in a boiling water-bath. The dissolved agarose was poured on to a glass microscope-slide and allowed to gel. A small well was cut into the centre of the gel and filled with rabbit serum. The slide was placed on the water-cooled floor of the apparatus shown in Fig. 1. (Paigen, 1956). Electrical contact between the ends of the gel sheet and the electrode vessels, filled with tris buffer, was made with paper wicks previously soaked in the same buffer. A current producing a voltage drop of 7V/cm. across the

Fig. 1.

Electrophoresis apparatus.


microscope slide.
agarose gel.
paper wicks.
water inlet.
water outlet.
WATER COOLED PLATFORM.
paper wicks.
carbon electrode.
buffer
rubber stopper.
ELECTRODE VESSEL.
ELECTRODE VESSEL.

length of the gel sheet was applied for 30 min. After staining with nigrosine for 20 min. the slide was washed with 2% ($^{w}/_{v}$) CH_3COOH, and the distance the γ globulin had migrated towards the negative end of the slide was measured. In a perfectly neutral gel, discounting any side-effects, electroendosmosis should be absent. This degree of purity was not obtained, but the back flow of γ globulin was so slight (1-2 mm.), that the agarose was considered a good medium for electrophoresis.

Gel precipitin tests were done according to the method of Mead (1962a). As only small quantities of the antigen and the antibody were available, the tests were restricted to a micro method.

A 1% ($^{w}/_{v}$) solution of agar or agarose was prepared in 0.85% ($^{w}/_{v}$) saline. The hot solution of agar was poured into a petri dish containing a small quantity of thiomersalate, to a depth of approximately 1-2 mm. Thiomersalate was not added to the agar solution while it was being dissolved as it was heat sensitive. After the solution had gelled a hexagonal arrangement of wells was cut in the gel with two cutters producing wells 2 mm. in diameter but of different spacing. The first cutter produced wells with centres 5 mm. apart (narrow spacing), and the second, wells with centres 7.5 mm. apart (wide spacing). The centre well within

the hexagonal was filled with concentrated antiserum, the outer wells with antigen, and the dish transferred to a desiccator with a water-saturated atmosphere produced by placing wet cotton wool at the bottom of the apparatus.

After the lines of precipitation had developed contact photographic prints were made. After this the agar was washed with thiomersalate saline for three days to remove residual unprecipitated protein, before the plates were stained with nigrosine and washed with 2% ($^{v}/_{v}$) CH_3COOH and contact prints were again made.

Spectrophotometry. A Unicam SP 500 instrument was used throughout these investigations. Silica micro and 1 cm. quartz cells were used for ultraviolet measurements, and glass micro, 1 cm. glass and 2 cm. glass cells for colourimetric measurements. All solutions were read against a blank of either water or the solvent in which the solution was prepared.

Exclusion chromotography. Initially columns were packed with granulated agar or agarose prepared as described by Polson (1961). However, with the introduction of the pearl form of agarose, Hjerten (1964), the former method of preparing agarose for use in exclusion chromatography was abandoned in favour of the new technique which gave better separations of the partially purified antigen solution.

Pearls were prepared according to Hjerten's (1964) method with minor modifications as follows. Four concentrations (3, 5, 7 and 10%) of pearls were prepared, but for the sake of simplicity a description of only the 3% will be given.

Agarose (9g) was dissolved in 300 ml. of water by heating over a water-bath with stirring. In a separate flask 470 ml. toluene, 130 ml. carbon tetrachloride and 1g Emulphor (Soda Fabricken, Germany) were well mixed and brought to 50°. A mechanical stirrer, (Kestner, London) was adjusted with a tachometer to rotate at 1,150 rev./min. in water. Once the agarose had dissolved, the stirrer was lowered into the agarose solution, the organic liquids were added and the solution was stirred for 1 min. Cold tap water was poured over the external surface of the flask to facilitate gelling of the spheres. After 5 min. of cooling the emulsion was transferred to a 300 mesh copper sieve and the organic liquids washed out with ether until the filtrate was clear. This modification of the original technique was introduced because the Buchner filter originally suggested for removing the organic liquids was blocked by the pearls. The pearls were suspended in 1 litre of water at 30° and the flask evacuated to remove the ether.

The pearls were then allowed to settle and the supernatant liquid was removed. The pearls were wet-sieved through copper sieves of varying diameter.

Glass columns (1.5x115 cm.) with sintered glass discs sealed into the wall immediately above the stop cocks were used. The column was filled with buffered saline A and sufficient glass ballotini added to form a layer approximately 1 cm. high on the sintered disc. This precautionary measure was introduced to keep the sintered disc clear of the pearls, which tended to obstruct the flow of the liquid through the column. After the ballotini had settled, the stop cock was opened slightly and a suspension of pearls in buffered saline A poured into the column. This procedure was repeated until the column had been packed to a height of 110 cm. During the packing the pearls were not allowed to settle completely to form a clear pearl-buffer interface, as this tended to pack the column in steps which interfered with the resolving power of the pearls. This was achieved by periodic stirring of the suspension in the column, using a glass rod. As the pearls had become contaminated with copper from the sieves, a solution of glycine (0.IM glycine pH 9.0) was passed through the column. A blue band, indicating

the presence of copper, was often noticed passing down the column and was run to waste prior to washing with large volumes of buffer.

Any irregularities in packing were revealed by a preliminary test using a mixture of rabbit haemoglobin and phenol red in buffered saline A. One ml. of this mixture was pipetted directly on to the surface of the pearls, allowed to drain into the column and washed down with small volumes of buffer before fitting a separating flask filled with buffer into the mouth of the column to produce a continuous flow of buffer.

The haemoglobin and phenol red bands tended to tail rather badly if the column was irregularly packed. However, columns homogeneously packed separated the two components into two compact distinct horizontal bands. Prior to their use for chromatography of the antigen, the columns were equilibrated with buffered saline A by allowing at least 1 litre to percolate through them.

The column effluent passed through an absorptiometer (Uvicord LKB-Produkter Fabriksaktiebolag, Sweden) and then to a fraction collector fitted with an event marker. The buffer used contained thiomersalate. This substance absorbs strongly at 254mμ, the wavelength of the filtered mercury light in the Uvicord. To

compensate for this effect buffer was allowed to run through the system for 2 - 3 hr., and the instrument was adjusted to ensure a constant and practical base line.

The Uvicord traces which were recorded on a graphic ammeter (Esterline-Angus Co., Ind., U.S.A.), represented the percentage absorption of eluting substances and were converted to graphs by plotting the extinction E against the fraction number. The extinction values were obtained by measuring the percentage transmission T at the mid point between marks indicating the emptying of the fraction collector syphon using the expression,

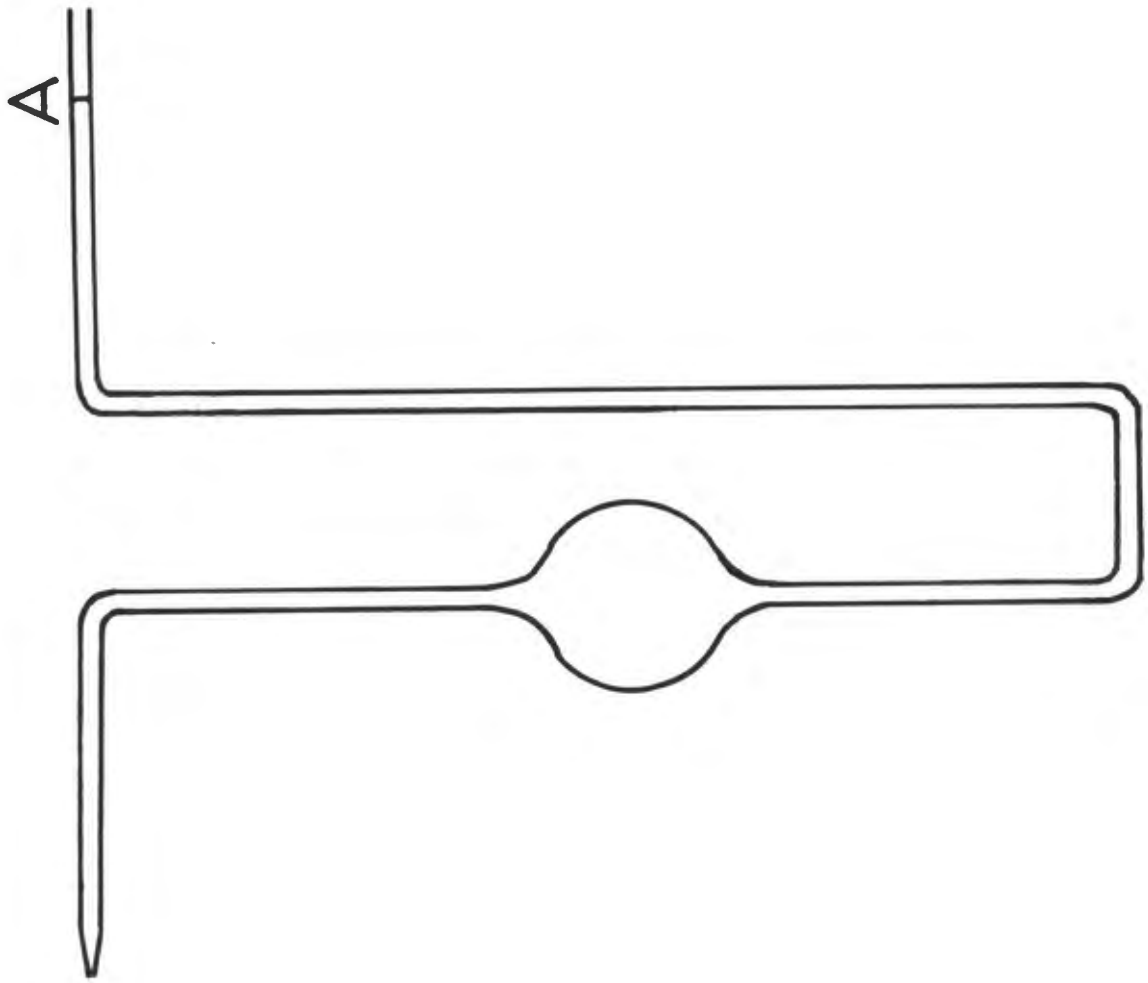
$$E = \log \frac{1}{T}$$

Pycnometry. Density determinations of solutions prepared with a 60% ($^{W}/_{V}$) solution of caesium chloride (CsCl) containing J. lalandii haemocyanin or the soluble antigen in 0.02M phosphate buffer pH 7.6 were done with a pycnometer constructed from pyrex glass (Fig. 2). The volume of the pycnometer was approximately 0.8 ml.

The apparatus was thoroughly cleaned in concentrated chromic acid, washed and then dried in an

Fig. 2.

Pycnometer used for determining densities.


A

oven at 120° to constant weight. It was filled with distilled water and immersed in a water-bath at 20°. After 15 min. at bath temperature, a piece of filter paper was applied to the fine end of the instrument and liquid withdrawn until the meniscus coincided with the mark A. The pycnometer was dried (external surface) and weighed filled with water. After being emptied and dried it was weighed again, and ultimately filled with the solution under investigation, and the procedure repeated.

Electron microscopy. Samples of purified antigen suspended in 0.02M phosphate buffer pH 7.6 were negatively stained by mixing with an equal volume of 2% ($^{w}/_{v}$) sodium tungstate in phosphate buffer (Valentine 1959). One drop of this mixture was applied to a carbon coated grid and after 30 sec. the surplus was removed with a micropipette. A piece of filter paper was applied to the edge of the grid to remove any excess fluid remaining. The grids were transferred to a calcium chloride desiccator for drying and were then examined and photographed in an EM3 Metropolitan-Vickers electron microscope.

Freeze-drying. Samples were freeze-dried in ampoules or round bottomed flasks and stored at 4° until used. The sample was snap frozen in a mixture of solid CO_2 and

acetone at -70°. The ampoule or flask which was exposed to the air was then attached to a condenser which was cooled by the same freezing mixture mentioned above. The condenser was attached to a "Speedivac" high vacuum pump (W. Edwards and Co., London) which evacuated air from the system. The pressure in the system was measured with a Pirani vacuum gauge (W. Edwards and Co., London). Samples were freeze-dried for approximately 17 hr. at a pressure of 0.01 mm. of Hg. On completion of the process air was let into the system slowly through a column containing silica gel, after which the ampoules or flasks were removed, and in the case of the ampoules they were sealed in a Chance flamemaster by pulling out at the centre of the stem. Flasks were closed with greased ground glass stoppers and the samples stored at 4°.

Dialysis. Cellulose dialysis casings (Visking Co., Chicago, U.S.A.) were thoroughly washed prior to their use. An appropriate length of the casing was cut from the roll and washed with distilled water. A knot was tied at one end of the casing and half filled with water. The water was shaken up and down for about 30 sec., run

out and replaced with fresh distilled water and the procedure repeated. The casings were left to soak in a beaker of water for 12-16 hr. and immediately before use were washed a final time as described above. The sample to be dialysed was poured into the bag which was closed by knotting the open end.

The washing and soaking procedure described above was introduced because inadequately washed bags contained impurities which absorbed fairly strongly in the ultraviolet light. This source of error was eliminated by the above treatment.

CHAPTER FOUR

EXTRACTION OF THE SOLUBLE ANTIGENS OF RABIES VIRUS

Introduction. The preparation of acid-precipitated purified (APP) extracts of infected and normal suckling mouse brains was described by Mead (1962a). Certain minor modifications were introduced by Katz, Larsson and Mead (1967), and are detailed below.

Method. Three-to-four-day-old suckling mice were inoculated intracerebrally in batches of about 200 with 0.02 ml. of a 10% ($^{w}/_{v}$) suspension of rabies infected suckling mouse brains in BS pH 7.0 containing 5% normal adult mouse serum. The infected brains were harvested three to four days after inoculation, when the mice were dead or obviously ill.

To each batch of brains (200) were added 75g of crushed distilled water ice and 225 ml. of cold BS pH 7.0. After homogenization in the blender for four half-minute periods, two drops of tributyl phosphate were added as an antifoam. The homogenate in 25 ml. portions was ultrasonicated for 5 min. per portion at 0°. After centrifugation at 44,000g for 2 hr. in the No 30 Spinco rotor, the supernatant fluid was withdrawn and transferred to

two cellulose dialysis sacs. Dialysis was continued for approximately 14 hr. at 4° against a solution consisting of 4 litres distilled water, 1 litre EDTA saline, 36g NaCl and 0.4g thiomersalate.

Acid precipitation. Hydrochloric acid (0.1N) was added slowly to the vigorously stirred extract in a beaker surrounded by ice until a pH meter, with its electrodes immersed in the fluid, indicated 4.5. The precipitate which began to form at a pH of about 5 was at once centrifuged in the precooled No 30 Spinco rotor at 8,700 g for 30 min. at 0°. The supernatant was discarded and the pellets were washed by suspension in acid saline pH 4.5 and centrifuged as before. The washing was repeated with about a 100 ml. of acid saline by alternate resuspension and centrifugation. The procedure was carried out as quickly as possible to minimize the destruction of the soluble antigens by the acid. The antigens were extracted from the washed precipitate by suspending it in 20 ml. tris buffered saline pH 8.4, and centrifuging as before. The cloudy supernatant fluid was removed completely and stored at 4°. The pellets were resuspended in tris buffer pH 8.4 and the suspension vigorously stirred for 1 hr. at 4° and again centrifuged. A third extraction was performed on the suspension by stirring

for 2 -3 hr. on this occasion. The combined extracts were dialysed against 5 litres of thiomersalate water (1/10,000 $^{w}/_{v}$) at 4^{0} for 14 hr. The dialysed supernatant fluid was centrifuged in the No 40 spinco rotor at 59,000g for 1 hr. Two thirds of the supernatant were removed and the remainder was transferred to a clean tube and recentrifuged as before. The clear supernatant from this centrifugation was added to the first and the mixture sampled for CFT and gel precipitin tests. The remainder was freeze-dried in a 250 ml. round-bottomed flask and stored at 4^{0}. Two hundred infected brains yielded about 60 ml. of pale yellow extract, which had a CF titre of about 300 - 600. A concentrated solution of the dried product usually gave three lines of precipitation in the gel precipitin test with 3x concentrated mouse antirabies serum as source of antibody.

The effectiveness of centrifugation in removing the virus (which acts as an antigen in complement fixation titrations and gel precipitin tests) was shown in infectivity tests done with the supernatant fluid obtained after centrifugation. Each centrifugation was capable of removing 99.9% of the virus.

In order to demonstrate the effectiveness of the purification procedure in the removal of substances

other than the antigen, normal suckling mouse brains were extracted in the same way. Concentrated extracts of these brains showed no lines of precipitation against 3x concentrated mouse antinormal mouse brain serum and against 3x concentrated antirabies mouse serum. No CF activity was found in these extracts at dilutions less than 1/100.

EXPERIMENTAL.

As mentioned previously, the large antigen was resistant to digestion with trypsin (Van den Ende, Polson and Turner 1957., Mead 1962a). However, the smaller antigens were sensitive to this enzyme, and it was felt that this characteristic could be used to advantage in ridding the large antigen of traces of the smaller components and protein impurities.

A feature of APP extracts was the marked absorption at 260mμ, which suggested the presence of nucleotide-like material. This absorption, which was initially thought to be due entirely to nucleotide impurity, led to experiments with nucleases, in the hope that the nucleotides would be degraded into fragments eliminable by dialysis or centrifugation.

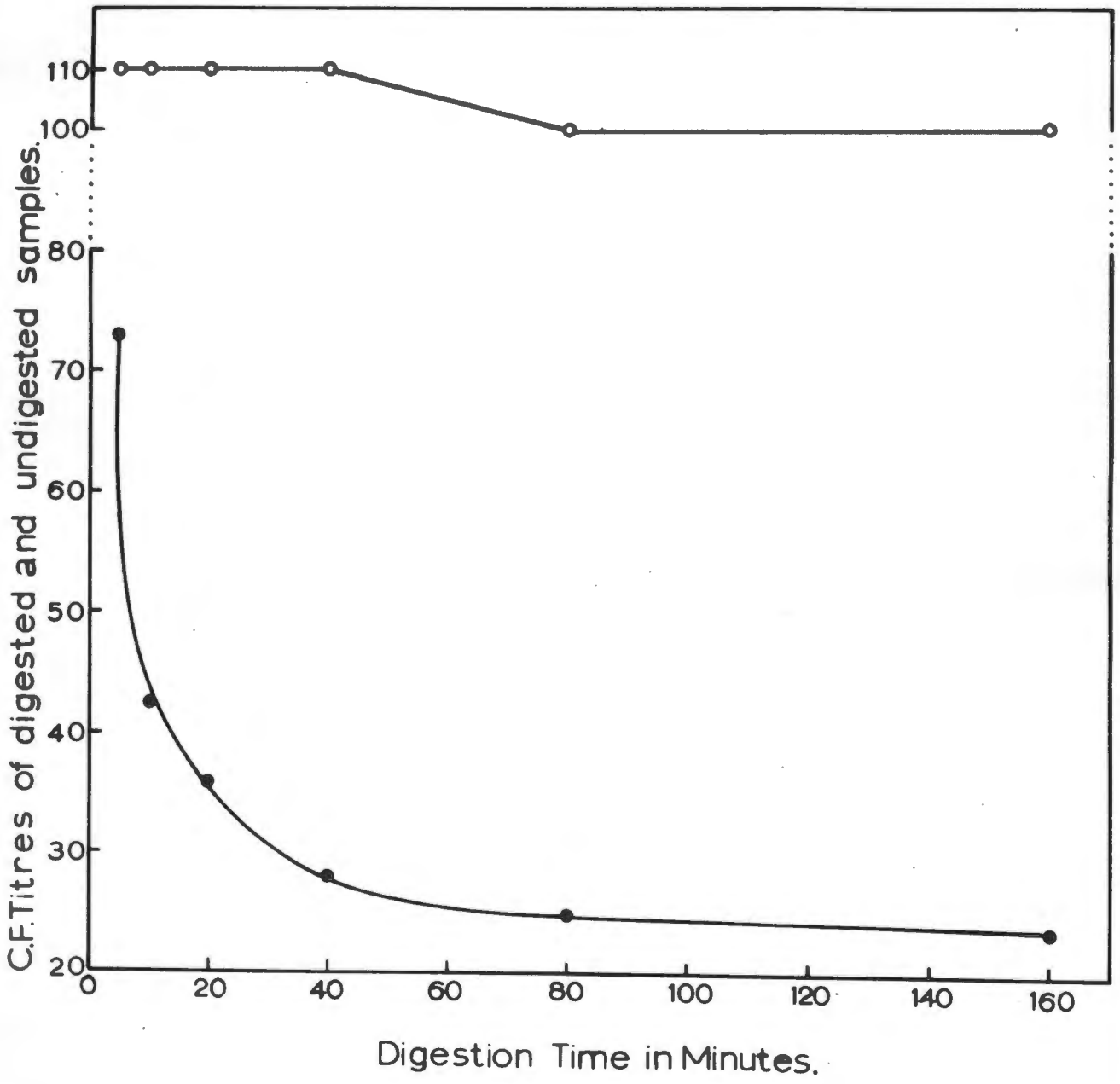
Enzymes. Ribonuclease (RNAase) 5x crystallized, salt and protease free; deoxyribonuclease (DNAase) was salt free with 36% activity of the crystalline enzyme; trypsin 2x crystallized and salt free; α chymotrypsin 3x crystallized and salt free, were obtained from Seravac Laboratories, Cape Town. Trypsin inhibitor (soybean) 5x crystallized was obtained from General Biochemicals, U.S.A.

Treatment with trypsin and chymotrypsin. The sensitivity of the "inner" and probably the "intermediate" antigens to trypsin was reported by Mead (1962a), and Katz, Larsson and Mead (1967).

An experiment was performed to confirm the immunity of the "large" ("outer") antigen to this treatment. The freeze-dried residue from an APP extract from infected brain material (originally 10 ml.) was dissolved in 20 ml. 0.IM tris buffer pH 7.6, and 10 ml. portions pipetted into each of two test tubes. To the experimental tube was added 10 mg. trypsin and both were immersed in a water-bath at 37°. At 5, 10, 20, 40, 80 and 160 min. intervals, 1 ml. samples were removed from each tube and transferred to test tubes containing 1 mg. trypsin inhibitor. The contents of each tube were well mixed and kept at 4° until titrated. The results (Fig. 3) were obtained by plotting the CF titres of the two solutions against the digestion time in min. As approximately 77% of the CF activity had disappeared in the experimental tube during digestion it was assumed that the remaining 23% was due to the large antigen. The slight drop in titre between the 80 and 160 min. intervals of the experimental and control solution was probably not significant, as both

Fig. 3.

Change in CF titre of an APP extract during digestion with trypsin. The control solution (○——○) in 0.1M-tris buffer pH 7.6, and the experimental solution (●——●) same as the control but containing trypsin (1mg./ml.). Both solutions were kept at 37°; samples taken at intervals were treated with trypsin inhibitor (1mg./ml.).


C.F.Titres of digested and undigested samples.
110
100
80
70
60
50
40
30
20
0
20
40
60
80
100
120
140
160
Digestion Time in Minutes.

these solutions had been titrated the day after the experiment.

A similar experiment with chymotrypsin was undertaken. The freeze-dried residue of an APP extract from infected material (originally 5 ml.) was dissolved in the same volume of 0.1M tris buffer pH 7.6, and four tubes each containing 1 ml. of redissolved extract were treated as follows. To each of two tubes was added 1 mg. of chymotrypsin, the remaining two had no enzyme. One tube with enzyme and one without were kept at 37° for 1 hr. The remaining pair were kept at 4°. After this period, the contents of each tube were diluted 1/100 with CFT saline andtitrated.

The titres of the "cold" and "hot" tubes containing no enzyme were 600 and 475 respectively, and the "cold" and "hot" enzyme treated samples were 230 and 100 respectively. Unfortunately the interpretation of the results was complicated by the drop in titre of the "hot" untreated sample, but it seems probable that the enzyme was responsible for at least part of the loss of CF activity.

In an attempt to confirm this assumption, a similar experiment to that described above was repeated, but the "hot" and "cold" samples from enzyme and non-enzyme

treated tubes were applied to an Ouchterlony plate at 5, 30 and 90 min. intervals after the addition of enzyme. To avoid any possible interference of residual chymotrypsin with the antirabies serum, used as a source of antibody, the agarose plate was prepared in the presence of β phenylproprionic acid, a known chymotrypsin inhibitor (Bettelheim and Neurath 1955). The agarose was dissolved (1% $^{w}/_{v}$) in a solution containing 0.05M-Na_2HPO_4, 0.02M-NaCl and 0.1Mβ-phenylproprionic acid and the pH adjusted to 7.0 with 2N-NaOH. The inner and outer lines were visible in the "hot" and "cold" solutions containing no enzyme, but the inner line had disappeared after 30 min. digestion in both the "cold" and "hot" enzyme-treated samples, but was still present after 5 min. digestion.

Treatment of APP extracts with RNAase and DNAase. The freeze-dried residue from an APP extract from infected brains (originally 5 ml.) was redissolved in 5 ml. 0.2M tris buffer pH 7.6 and clarified by centrifuging at 6,590g for 20 min. Portions (0.8 ml.) were transferred to 5 previously washed dialysis sacs. Two sacs received 0.4 ml. of aqueous RNAase solutions giving final concentrations of 10 and 100µg/ml. of the enzyme. Two sacs received 0.4 ml. of aqueous DNAase solutions to give a final concentration of 10 and 100µg/ml. of the enzyme and a further 0.4 ml. of

of aqueous $MgSO_4$ to give a final Mg concentration of 0.005M. The control sac received 0.4 ml. of $MgSO_4$. The sacs were surrounded by 1.6 ml. 0.1M tris buffer pH 7.6 and kept for 1 hr. at 38°. They were then dialysed separately against 200 ml. 0.1M tris buffer for 4 hr. at 4° and then against distilled water for 14 hr. at 4° in order to remove the digestion products by dialysis.

The solutions were diluted to 4 ml. with distilled water and the absorption measured against a distilled water blank in the spectrophotometer between 230 and 300mμ. The samples were then freeze-dried and redissolved in 0.05 ml. CFT saline and analyzed immunologically by the Ouchterlony technique.

Enzyme treatment of APP extracts from infected material revealed interesting facts besides substantiating the findings of Van den Ende, Polson and Turner (1957), and Mead (1962). The experiment with DNAase and RNAase showed that neither enzyme had any effect on the soluble antigens. Treatment with RNAase, followed by dialysis did cause some reduction in absorption at 260mμ probably indicating partial removal of nucleotides. Furthermore the ultraviolet absorption curves of the five samples in this experiment had maxima at 260mμ and minima at 245mμ,

but the extinctions at 260 mμ of the solutions treated with RNAase were lower than those of the control and DNAase treated solutions. Each solution showed two lines of precipitation in the Ouchterlony test corresponding to the "inner" and "outer" antigens described by Mead (1962a) and Katz, Larsson and Mead (1967).

Valentine and Isaacs (1957a) showed that influenza A virus was susceptible to trypsin digestion only after preliminary treatment with 0.1N-HCl, and to RNAase after trypsin treatment. An APP extract from infected material, freed of small antigens by centrifuging, was divided into two 1 ml. portions. To the experimental tube was added 1 ml. of 0.1N-HCl. Thirty seconds after the addition of the acid 3 ml. 0.4M-Na_2HPO_4 was added to adjust the pH to 7.6. To the control tube containing 1 ml. of antigen was added 4 ml. of a solution prepared by mixing 0.1N-HCl and 0.4M-Na_2HPO_4 to obtain a pH 7.6. Both tubes were sampled (0.4 ml.) for CF titration.

From the results it seemed that exposure to acid for 30 sec. destroyed the complement-fixing activity of the soluble antigen prior to the addition of enzymes.

The APP extracts of normal and infected brains were subjected to enzyme treatment as a step in their purification, and as the RNAase digestion preceded that

of trypsin it was of interest to determine the effect of RNAase on a trypsin-digested APP extract. It was thought that the trypsin might induce a change in the soluble antigen thus rendering it sensitive to RNAase digestion.

In an experiment designed to test this assumption, 3 ml. of an APP extract obtained from infected material was mixed with 3 mg. trypsin and kept at 37° for 90 min. After this period, 3 mg. of trypsin inhibitor was added, well mixed, and a sample (0.5 ml.) was removed and diluted to 10 ml. with CFT saline and kept at 4° until titrated. The remainder was divided into two 1 ml. portions, one tube receiving 0.2 mg. RNAase, and both were kept at 37° for 1 hr. Both solutions were diluted 1/20 with CFT saline and all three titrated. No change was detected in the CF titres of the three solutions. This showed that the sequence of RNAase and trypsin digestion were not important.

Discussion. Normal and infected suckling mouse brains after acid precipitation and alkaline extraction yielded extracts which were partially purified. The normal brain extract had no complement-fixing activity at dilutions greater than 1/100, and no lines of precipitation were observed in Ouchterlony plates tested with concentrated mouse antirabies serum. The infected material gavethree lines of precipitation in agarose, and at a volume of approximately 60 ml.

had a complement-fixing titre between 300 and 600.

Trypsin destroyed the inner and possibly the intermediate antigens, but the large (outer) antigen was completely immune to digestion by this enzyme. Chymotrypsin, an enzyme of a different specificity, destroyed the inner and intermediate antigens, but had no effect on the large antigen.

During digestion of APP extracts with RNAase and trypsin, it was found that the sequence of digestion with these enzymes was not important as the large antigen was resistant to their effects irrespective of the sequence.

The final experiment, which consisted of treating the large antigen with 0.1N-HCl, showed thatthe antigen was destroyed immediately even prior to the addition of RNAase and trypsin.

It, therefore, seems probable that all the antigens present in infected brain extracts were resistant to RNAase and DNAase digestion, and only the large antigen was resistant to trypsin and chymotrypsin, but not to the effects of 0.1N-HCl.

CHAPTER FIVE

THE PURIFICATION OF THE LARGEST RABIES SOLUBLE ANTIGEN

Introduction. These experiments were based to a large extent on the observations from the preceeding chapter. Firstly, it was shown that centrifugation in the J rotor showed an almost complete separation of the large antigen from the smaller antigens. Complete removal of the smaller antigens was achieved by digestion with trypsin. Digestion with RNAase and DNAase was also done at this stage.

Repeated ultracentrifugation alone of the digested product failed to purify the large antigen completely, as was demonstrated by the persistence of substances in the extract of normal brains treated in a similar manner.

Exclusion chromotography of the infected extract separated the large antigen from components appearing to have larger and smaller particle sizes. Nothing was detected in the corresponding fractions from normal brain extracts treated in a similar manner.

As the large antigen solution, purified as described, continued to absorb maximally at 260mμ in the ultraviolet, density gradient centrifugations were introduced to remove any free high molecular weight nucleic

acid which might have escaped the enzyme treatment. Degraded nucleotides and other impurities of low molecular weight were presumably removed by exclusion chromatography.

Analytical ultracentrifugation provided a check on all the operations undertaken with the large antigen, for its boundary andthose of any impurities were visible during centrifugation with the Schlieren optical system. Methods. Preparative centrifugations were done in a model L Spinco centrifuge at forces (measured at the centre of the tube) between 4,000g and 95,000g in the No 30 and No 40 rotors, and in the SW 39 swinging bucket rotor. A refrigerated MSE machine was used for centrifugations not exceeding 3,000 rev./min.

Preparative centrifugations (at forces greater than 95,000g) were performed by Dr T.H. Mead in a model E Spinco ultracentrifuge using the "J" rotor at a centrifuging force of 161,180g (Katz, Larsson and Mead 1967).

As the "J" rotor could be maintained at a fairly constant temperature during centrifugation (to reduce convection currents), the temperature of maximum density of certain salt solutions used as suspension media was determined. The machine could, therefore, be set to the appropriate temperature at which the density of the solution under investigation was the greatest, so reducing the effects

of convection to a minimum. A dilatometer of approximately 750 ml. capacity was used for these measurements. The instrument was filled with a salt solution of known concentration and immersed in a vigorously stirred thermostatically controlled water bath cooled by the circulation of refrigerated alcohol through copper coils suspended in the bath. Readings of the bath temperature and the position of the meniscus in the capillary of the dilatometer were noted (once temperature equilibrium had been reached) at 30 sec. intervals for 10 min., at ten different temperatures rising by 0.5° from 1.5° to 6.0°. The dilatometer was then washed and refilled with de-aerated distilled water, and the procedure repeated. As the values obtained for the maximum density of water during these experiments varied between 4.8 and 4.82 (this increment of 0.8 was attributed to the change in volume of the instrument and was subtracted from each reading.) The corrected temperatures of maximum density of the salt solutions were; 0.I4M-NaCl 2.12°, 0.07M-NaCl 3.0°, buffered saline A 1.88° and 0.14M sucrose 1.67°.

Sedimentation of the large antigen was performed in the "J" rotor at 161,180g for 4 hr. at temperatures varying between 1.5° and 2.5° to reduce convection. However, it was found necessary to add a little washed

Hyflo-supercel to each tube prior to centrifugation in order to trap the pellet, so allowing the supernatant fluid to be removed without disturbing the pellet (Katz, Larsson and Mead, 1967).

Acid-precipitation-purified extracts were centrifuged prior to enzyme treatment to separate the large antigen from the smaller antigens and low molecular weight impurities which remained in the supernatant fluid.

Gradient iso-density centrifugation. Banding of substances with small sedimentation coefficients like the large antigen would have been too slow with the force available in the S.W. 39 rotor. It was, therefore, found necessary to carry out the density gradient centrifugation in the analytical rotor. To enable the antigen containing band to be recovered the separation cell of Tiselius, Pederson and Svedberg (1937) was used. The cell consisted of a Kel-F centrepiece with a fixed partition of perforated plastic which supported a piece of filter paper of the same dimensions. Two centrifugations at different initial densities were necessary to rid the large antigen of more and of less dense impurities. For the first centrifugation a density was chosen which banded the substance to be purified either above or below the partition, and as close to it as possible. The solution containing the

substance under investigation was then removed from the cell, and the density altered so that it banded on the opposite side of the partition during the second centrifugation.

Iso-density centrifugations were performed by Dr T.H. Mead in an An-D rotor of a Spinco model E ultracentrifuge. Jasus lalandii haemocyanin, which has a sedimentation constant and a buoyant density in CsCl very close to that of the antigen, was used in preliminary experiments (Katz, Larsson and Mead 1967). The haemocyanin was diluted (1/20) with 0.02M phosphate buffer pH 7.5, and a 60% ($^{w}/_{v}$) solution of CsCl added to give the desired densities, which were determined pycnometrically at the temperature of centrifugation (20° or 22°). Centrifugations were done at 161,180g for 5 - 6 hr.

Experimental.

Purification of the large antigen. Acid-precipitated-purified extracts of 613 infected suckling mouse brains and approximately the same number of normal brains underwent the same treatment, but for simplicity a description of the infected brain material only will be given.

The freeze-dried residue from 613 brains was dissolved in 24 ml 0.2M tris buffer pH 7.6. The solution

(24 ml.) was well mixed and sampled for CF titration, after which it was centrifuged for 4 hr. at 161,180g and 2.5° in 4 "J" rotor tubes each containing 0.2g of Hyflo-super-cel. The supernatant fluid was removed completely and the four pellets and Hyflo suspended in 2.5 ml. tris buffer and transferred to a conical glass centrifuge tube. The mixture was centrifuged for 10 min. at 2,500 rev./min., and the supernatant fluid removed; the pellet extracted a further 6x in a similar manner with small amounts of tris buffer until the total volume was 12 ml. The solution was sampled for CF titration, and this showed that 78% of the original CF activity had been recovered from the pellets. It was of interest that whereas considerable losses of antigen occurred during most stages in the purification, the first preparative centrifugations frequently gave an apparent recovery exceeding 100% based on CF titre x volume. It seemed that one or more of the antigens could fix more complement when the largest antigen was separated from the others. The solution containing the large antigen (and the corresponding extract from the normal brains) was digested at 37° with RNAase (0.5mg./ml.), DNAase (1mg./ml. after the addition of 4.9mg. $MgSO_4.7H_2O$/ml.) and trypsin (1mg/ml.). Each enzyme was allowed to react for 45 min. before the addition

of the next enzyme; 45 min. after the addition of trypsin, trypsin inhibitor (1mg./ml.) was added to the turbid solution. The solutions were centrifuged for 40 min. at 6,590g in the No 40 rotor, $^{2}/_{3}$ of the supernatant were removed and the remainder transferred to a clean tube and recentrifuged. The pellets remaining after the second centrifugation were extracted with the aid of a small volume of tris buffer and the SNF from the third centrifugation was pooled with the previous SNF's (volume 12 ml.). CF titration showed that 61% of the activity of the infected extract was recovered after digestion.

The solution was dialysed against buffered saline A, diluted to 18 ml. and centrifuged in 3 "J" rotor tubes each containing 0.15g. Hyflo at 161,180g and 2.5° for 4 hr. The supernatants were completely removed and the Hyflo and pellets extracted as before with 6 portions of 0.2M tris buffer to a total volume of 9 ml. The recovery (by CF titration) was 60%. The solutions were concentrated by "perfrigeration" to $\pm$3 ml., dialysed against water and freeze-dried.

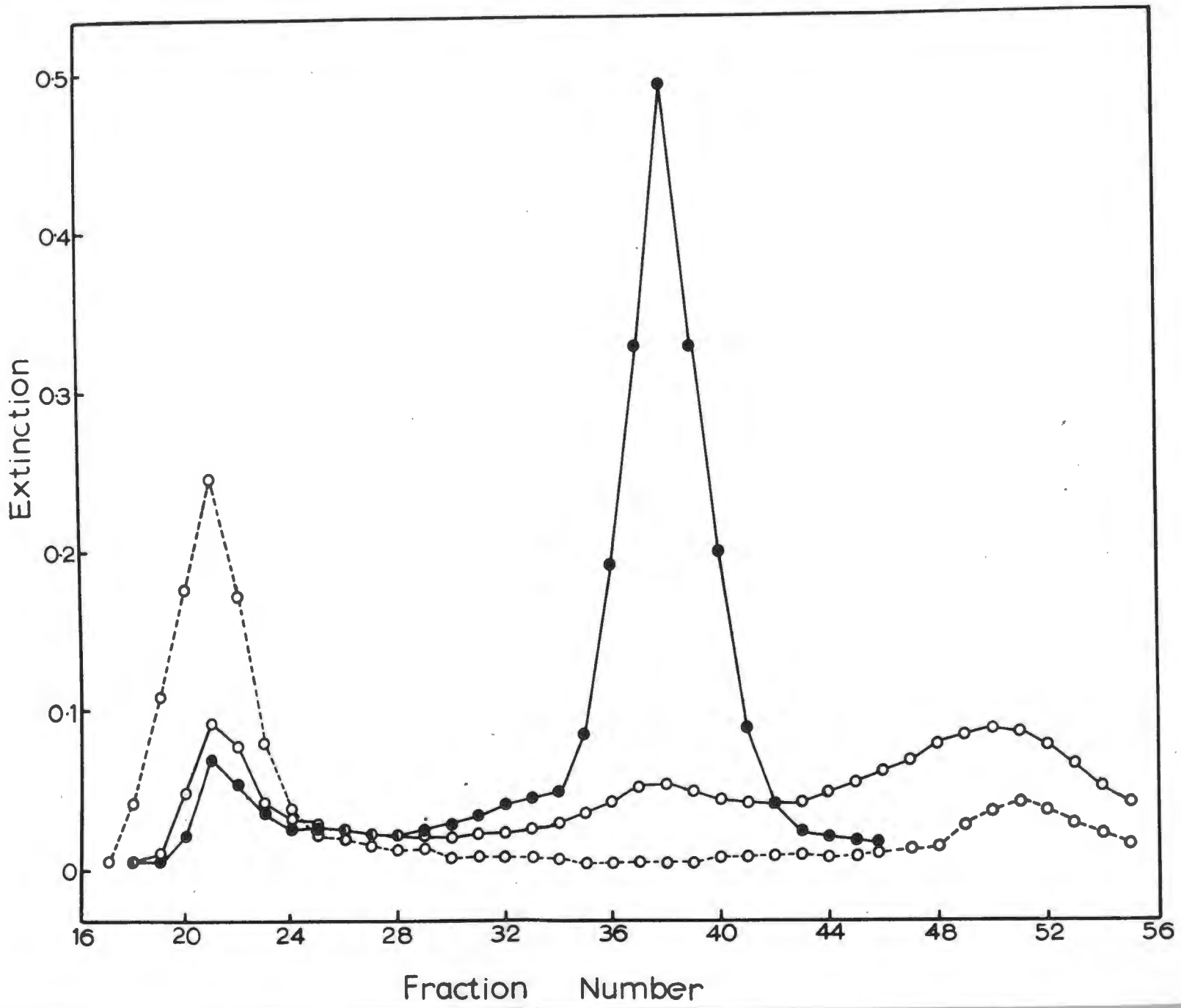
The freeze-dried residues (normal and infected) were dissolved in 1 ml. buffered saline A and were applied successively to a column (1.5 x 100 cm.), containing 7% agarose pearls, which was fed with the same solvent. The effluent was collected in 2.75 ml. fractions after

having passed through the Uvicord, fraction one being taken during the application of the sample.

The traces obtained during exclusion chromatography of extracts of normal and infected brains and of a sample of Jasus lalandii haemocyanin, dialysed against buffered saline A, are shown in Fig. 4. Previous experiments with this column, (Katz, Larsson and Mead 1967), showed that the small peak between fractions 32 and 40 contained the antigen which was confirmed by CF titration. Consequently, these fractions were pooled for both the normal and infected extracts during this experiment. The CF titration showed a 35% recovery. The pooled fractions were centrifuged in 5 "J" rotor tubes each containing 0.04g. Hyflo for 4 hr. at 161,180g and 2.5°. The SNF's were removed and discarded and the pellets and Hyflo extracted to a total volume of 2 ml. (recovery 52%) and the samples reapplied to the column. Fractions 33 to 41 were pooled (recovery 77%) and recentrifuged as before in "J" rotor tubes containing 0.03g. Hyflo. The Hyflo and pellets were extracted to a total volume of 2 ml. (recovery 48%). Both the normal and infected extracts were dialysed against 0.02M phosphate buffer pH 7.5 to remove thiomersalate before examination in the spectrophotometer. A sample (2 ml.) of buffered saline A

Fig. 4.

Exclusion chromatography on a 1.5 x 100 cm. column of agarose pearls of J. lalandii haemocyanin (●——●), digested and centrifuged extracts of normal brains (○--------○), and a similar extract of infected brains (○——○). Plot of extinction against fraction number.


0·5
0·4
0·3
0·2
0·1
0
Extinction
16
20
24
28
32
36
40
44
48
52
56
Fraction Number

which had been dialysed under identical conditions was used as a blank for the spectrophotometry. The ultra-violet absorption curves of the infected and normal brain extracts are represented in Fig. 5.

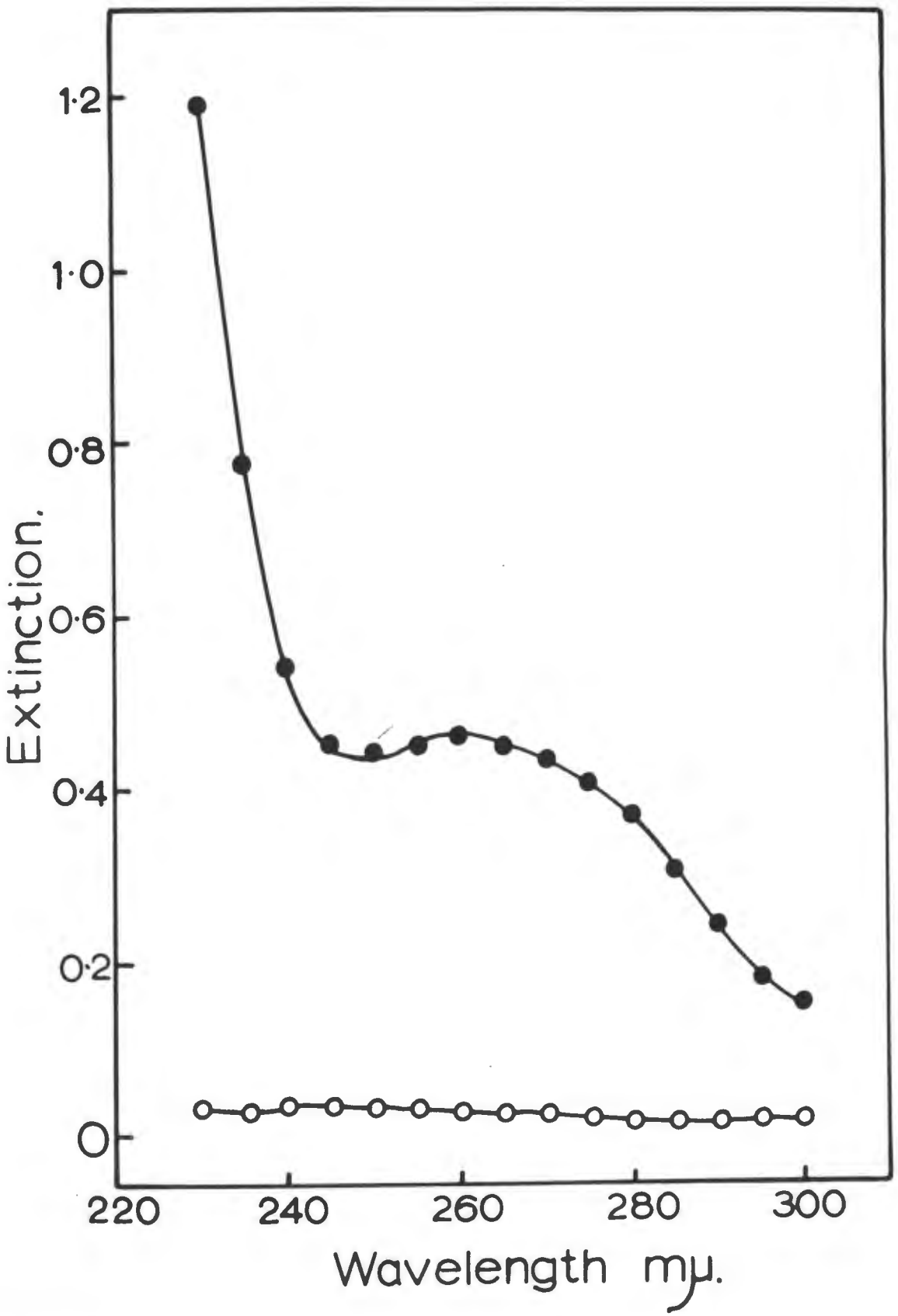
Flow diagram for the purification of the antigen.

APP extracts of normal and infected brains ——→ centrifuged 161,180g for 4 hr. pellets extracted and SNF's from extraction ——→ enzyme digestion ——→ centrifuge 6,590g for 40 min. dialyse, centrifuge 161,180g for 4 hr. extract pellets perfrigerate, dialyse, freeze-dry redissolve, exclusion chromatography, fractions 32 - 40 pooled ——→ centrifuge for 161,180g, extract pellets ——→ exclusion chromatography fractions 33 - 41 pooled ——→ centrifuge 161,180g extract pellets, dialyse ——→ freeze-dry.

In a subsequent experiment the freeze-dried extract of infected brains received the initial treatment described above and summarized in the flow diagram. The freeze-dried residue dissolved in 0.85 ml. 60% ($^{w}/_{v}$) CsCl solution and well mixed. The density of the solution was determined pycnometrically and was found to be 1.343g./cm.^{3} at 20°. After centrifugation for 5.5 hr. at 187,000g and 20° in the fixed partition cell, only one Schlieren band was detected, but the solutions from above and below the partition were removed and dialysed for 2 days against

Fig. 5.

Ultraviolet absorption curves of APP extracts of infected (●——●) and normal brains (○——○) after enzyme treatment and two cycles of exclusion chromatography on 7% agarose pearl. Both solutions were read against a blank prepared by dialysing buffered saline A under the same conditions as the samples in order to remove thiomersalate.

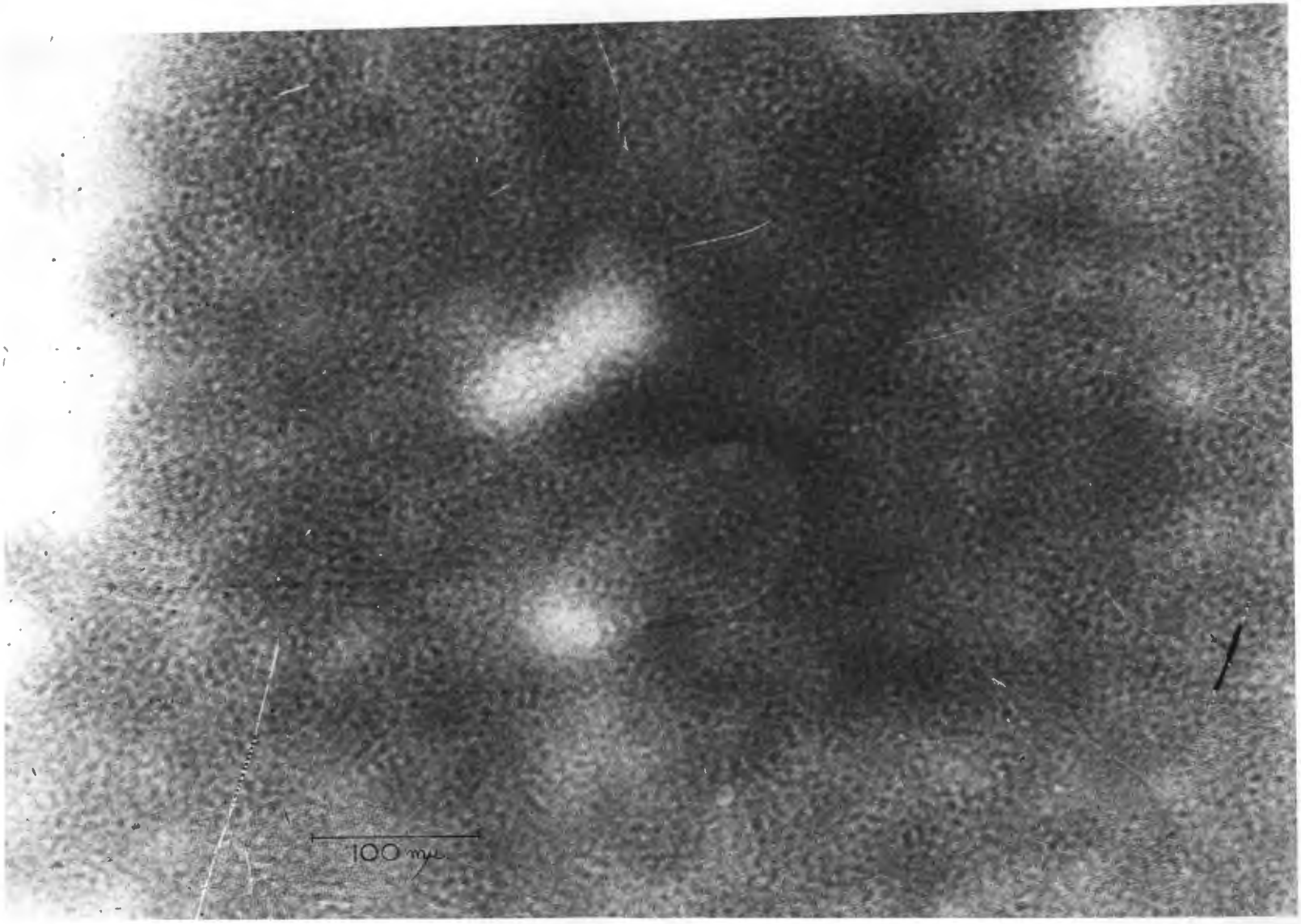

1·2
1·0
0·8
0·6
0·4
0·2
0
Extinction.
220
240
260
280
300
Wavelength mμ.

phosphate buffer and for one day against water. On examination in the spectrophotometer both solutions had clearly defined maxima at 260mμ, but most of the material was in the upper part of the cell. The solution from above the partition was freeze-dried and redissolved in buffered CsCl solution to give a density of 1.314g./cm.^{3} determined pycnometrically at 20°. After centrifugation at the same time and velocity the band unfortunately coincided with the partition, and consequently the contents were withdrawn and diluted to give a density of 1.304g./cm.^{3} and recentrifuged. During the third centrifugation there was no convincing evidence of a band, but the solutions from above and below the partition were treated as before. The ultraviolet absorption spectrum was very weak for both solutions; the sample below the partition had a maximum at 260mμ, and the one above a maximum at 270mμ. The sample from below the partition was freeze-dried and the residue dissolved in a drop of 0.02M phosphate buffer pH 7.8, and the sample negatively stained by Valentine's method (1959) and applied to carbon coated electron microscope grids and examined in the microscope. The appearance of the antigen is shown as a ring-like structure in Fig. 6.

In the hope of obtaining sufficient antigen for

Fig. 6.

Electron micrograph of the largest antigen purified by enzyme treatment, sedimentation, two cycles of exclusion chromatography on agarose pearls and density gradient centrifugation. Negatively stained with 0.02M-Na_2HPO_4 and sodium tungstate. Photograph is a positive.


100 mμ

nucleic acid and nitrogen determinations, 1,600 infected suckling mouse brains were purified as described in the previous chapters. Prior to purification the extract was divided into two equal portions and treated independently. They were recombined before the second cycle of exclusion chromatography.

The initial density gradient centrifugation at 1.308g./cm.^{3} showed two distinct bands in the partition cell, the upper band of impurity and the lower band of the antigen. The ultraviolet absorption spectrum of the solution removed from the upper part of the cell had a maximum between 265 and 270 mμ and that from below the partition at 260 mμ. The component from the lower part of the cell was dialysed, freeze-dried and recentrifuged in buffered CsCl of density 1.340g./cm.^{3} for 6 hr. A single band appeared in the upper part of the cell, but both the components were dialysed, titrated and examined in the spectrophotometer. At a dilution of 1/50 the sample from below the partition had no CF activity, but that from above had a titre of 300. This was a recovery of only 15% from the fluid taken from below the partition after the initial density centrifugation. The poor recovery may be attributed to the possible absorption of the antigen by the filter paper supported on the partition. The solution containing the antigen was

dialysed and freeze-dried and redissolved in ± 2 ml. 0.02M phosphate buffer pH 7.5, and kept at 4° in an atmosphere saturated with water vapour and chloroform. Samples were removed for analysis as required.

Discussion. Acid-precipitated-purified extracts of infected and normal suckling mouse brains were purified by the method of Mead (1962a) and Katz, Larsson and Mead (1967). Both extracts were subjected to an initial preparative centrifugation in the presence of Hyflo-super-cel, which permitted the removal of the supernatant fluid without disturbing the pellet. The supernatant fluid contained the small antigens (in the case of infected material) and low molecular weight impurities in both the infected and normal extracts.

Enzymatic treatment of the extracts was carried out in an attempt to reduce the size of the protein and nucleotide-like impurities, which were ultimately removed with the enzymes in a second preparative centrifugation, as the large antigen was resistant to this treatment.

Numerous experiments were done using DEAE cellulose, Sephadex G75 and G200, granulated agar and agarose and agarose pearls in an attempt to purify the large antigen (Katz, Larsson and Mead 1967). The 7% agarose pearls were found to be the most effective means

of purification during exclusion-chromatography experiments. The first peak impurity (Fig. 4) obtained on exclusion chromatography, and which was always cloudy in appearance, was common to infected and normal brain extracts and also to Jasus lalandii haemocyanin. This impurity was attributed to a substance or substances prone to denaturation or aggregation during chromatography, as the solutions applied to the column were always clear. The second peak (fractions 34 - 42) was present in infected samples but not in the normal extract, and it corresponded to the large antigen as shown by CF titration. The second peak seems to have separated rather well from a third peak of low molecular weight impurity which had no CF activity.

After a third preparative centrifugation and a second cycle of exclusion chromatography on 7% agarose pearls, the dialysed solutions were subjected to iso-density gradient centrifugation in the partition cell. This method was applied to rid the antigen of impurities of similar molecular weight, but of different density. With care the antigen and the impurities could be banded on opposite sides of the partition and consequently more dense and less dense particles than the antigen could be removed by varying the density of the solution prior to centrifugation.

Ultraviolet absorption spectra of dialysed samples, obtained from above and below the partition, had maxima at 270mμ for the impurity fraction, and 260-265mμ for the antigen. This suggested that the antigen is a nucleotide-like substance, possibly a nucleoprotein. The presence of protein could be inferred from the ultraviolet absorption curve and from the fact that the antigen was stained with nigrosine during electrophoresis experiments (Katz, Larsson and Mead 1967).

The electron micrograph shown in Fig. 6 revealed a compact mass of rings of single turn helices with a diameter varying between 8.7 and 12.4mμ. Structures containing more than a single helix were not seen.

Owing to considerable losses of antigen during the purification procedure, 1,600 suckling mouse brains infected with rabies virus were prepared in the hope of producing sufficient of the large antigen for nucleic acid and protein determinations. The finally purified product dissolved in ± 2 ml. of phosphate buffer had a titre of about 300. This specimen was considered sufficiently pure for the biochemical investigations which were undertaken and will be described in the following chapters.

CHAPTER SIX

NUCLEIC ACID ESTIMATIONS.

Introduction. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are high molecular weight polymers of nucleotides which are composed of purine and pyrimidine bases combined with phosphorylated sugar. In the nucleic acid molecule the nucleotide units are joined through the phosphate ester groups. In most instances RNA and DNA differ only in that the former has ribose as its sugar and the latter deoxyribose, and the pyrimidine base uracil which is present in RNA is replaced by thymine in DNA. These small but significant differences have been utilised in conjunction with certain other properties of the nucleic acids, in their estimation.

Isolation of nucleic acids from biological material. The material under investigation is finely ground and the nucleic acids isolated by the addition of either trichloracetic acid (TCA) or perchloric acids (PCA), both of which are strong deproteinizing agents. Another important deproteinizing agent used extensively for the isolation of nucleic acids from biologically active material e.g. viruses, is aqueous phenol. The three classical methods for

isolating nucleic acids are the Schmidt-Thannhauser (1945), Schneider (1945) and Ogur-Rosen (1950).

The Schmidt-Thannhauser (1945) method relies on the different sensitivities of RNA and DNA to alkaline hydrolysis. The extracted tissue residue is incubated overnight with warm dilute alkali, which hydrolyses the RNA to acid soluble nucleotides without similarly affecting the DNA. On acidification of the digest, the DNA is precipitated along with degraded protein, and most of the RNA nucleotides remain in the supernatant fluid.

The Ogur-Rosen (1950) method is done by isolating the RNA with cold TCA and extracting the DNA with hot PCA. The tissue residue remaining after the lipid extraction is treated with TCA for 18 hr. at 4°; this residue is then treated with PCA at 80° for 30 min. to ensure the complete removal of DNA.

The Schneider (1945) method relies on treating the lipid-free fraction by extracting the nucleic acid with dilute TCA at 90° for 15 min. The nucleic acids are split off as soluble products in the acid extract, and non-nucleotide phosphorus compounds remain attached to the protein residue.

Hydrolysis of the nucleotides may yield nucleosides or free bases, sugars (ribose or deoxyribose) and

phosphoric acid. However pyrimidine nucleotides resist hydrolysis under normal conditions and only the purines are liberated. These products of hydrolysis afford the investigator three specific procedures for the determination of nucleic acids. The three most common methods are (a) the ultraviolet absorption of the bases, (b) the estimation of the phosphates and (c) the colourimetric estimation of ribose and deoxyribose. The first two methods do not differentiate between RNA and DNA, but the third is usually specific for one or the other sugar.

Determination of purines and pyrimidines by ultraviolet (UV) absorption measurements.

All nucleic acids absorb in the ultraviolet light between 260 and 265mμ, and it has been shown that this absorption is due to the purine and pyrimidine bases. As a method of estimating nucleic acids the UV technique has the advantage of being specific for these acids. The absorption is higher than that of proteins, which absorb at 275 - 280mμ. A small protein contamination in the nucleic acid solution will, therefore, be relatively unimportant.

Difficulties arise due to the fact that the absorption of the nucleic acids will vary according to the base composition, and secondly the absorption will depend on any degradation that the nucleic acid may have undergone

during extraction, as hydrolysis leads to an increase in absorption at 260mμ. For precise work, nucleic acids can be hydrolysed by acid to a mixture containing free purine and pyrimidine nucleotides. This mixture can be separated by electrophoresis or chromatography and the individual bases or their nucleotides can be estimated precisely by spectrophotometry.

As most nucleic acids are isolated from tissue with either TCA or PCA, certain precautions must be taken when estimating these nucleic acids by ultraviolet absorption measurements. Logan, Mannell and Rossiter (1952) have noted that TCA absorbs strongly in the UV region, and consequently all the standards and blanks must contain the same concentrations of TCA. Furthermore, they pointed out that the effect of heating TCA at 90° for 15 min. caused it to breakdown as follows.

$$CCl_3COOH \longrightarrow CHCl_3 + CO_2$$

This causes a change in absorption, and all the solutions under investigation must be heated for the same length of time. Both the extinction and wavelength of the maximum absorption of nucleic acids vary with the pH of the solution.

Fleck and Munro (1962) found that considerable amounts of protein were released with the RNA fraction from rat liver on prolonged digestion in alkali using the

Schmidt-Thannhauser method. Large errors were introduced in the UV absorption measurements by this protein by-product, and these could not be adequately corrected by taking readings at two wavelengths and applying corrections. They found, however, that by digesting in the presence of weak alkali for 1 hr. at 37° the amount of interfering protein liberated was negligible, and the recovery of RNA was not hindered by this modification.

Estimation of nucleic acids by the determination of phosphate. It is customary to estimate the total phosphate content of an extract, followed by the estimation of the inorganic phosphate. Organic phosphoprotein (non-nucleotide) must be removed with TCA, and similarly phospholipids must be removed with lipid solvents prior to estimating the nucleotide phosphate content, otherwise erroneously high readings will be obtained.

The improved method of Berenblum and Chain (1938) for the estimation of phosphates consists of extracting the reduced phosphomolybdic acid with isobutyl alcohol, in which it is extremely soluble. The alcohol extract obtained is shaken with an acidified aqueous solution of stannous chloride which reduces the phosphomolybdic acid to a blue complex.

The method outlined by Allen (1940) is as follows. For phosphate determinations in tissue, the material is gently heated in the presence of perchloric acid until the solution is colourless; a few drops of hydrogen peroxide may be added to facilitate the digestion. After cooling, the contents are transferred to a flask to which is added the reducing agent, in this case amidol, and the molybdate.

Colourimetric methods for the estimation of ribose.

Colourimetric procedures for the determination of ribose include reactions with orcinol, phloroglucinol, aniline, carbazole and cysteine (Hutchinson and Munro, 1961).

The mechanism of the reaction is as follows; the ribose is converted to furfural or furfural derivatives under controlled conditions in order to minimize similar conversions of other sugars. The furfural or its derivative is treated with any of the above-mentioned chromogenic substances, and the colours which develop are measured by appropriate instruments employing standards of known concentration. This reaction is complicated by numerous factors which tend to make these colour reactions unreliable.

Daly, Allfrey and Mirsky (1950) have noted that purine-bound ribose reacts more readily than pyrimidine bound. They found that the action of concentrated acid

at relatively high temperatures was necessary to liberate the pyrimidine ribose. Acid hydrolysis as is normally employed liberates the ribose from purine but leaves that in the pyrimidine unattacked and inaccessible for reaction with the chromogenic substance. Hutchinson and Munro (1961) have found that the pyrimidine ribose is not completely unreactive under normal test conditions, for it has been observed to give a colour representing from 3 - 10% of the total ribose.

Massart and Hoste (1947) claimed that they obtained full reaction from pyrimidine bound ribose by first brominating the pyrimidine ring before allowing the nucleotides to be treated with orcinol.

Many analyses for the estimation of individual bases have shown that the ratio of purine to pyrimidine is very different in nucleic acids obtained from different species. Therefore the value assigned to the result from any of the carbohydrate reactions will depend on the proportion of purine to pyrimidine nucleotides in the nucleic acid molecule. This problem can be circumvented by employing a standard of pure nucleic acid isolated from the tissue on which the determinations are carried out.

Finally, most of the carbohydrate reactions are not completely specific. Although the above discussion

may not engender a feeling of confidence towards employing these techniques for nucleic acid estimation, a certain degree of success may be obtained by controlling the conditions rigorously. The results may then be accepted with a fair amount of confidence.

The orcinol reaction depends upon the hydrolysis of RNA by acid at 100° to yield furfural from the ribose; this then is treated with the orcinol to give a green pigment.

In the original procedure of Dische and Schwarz (as cited by Hutchinson and Munro 1961), the final concentrations of the reagents was; 7.7N HCl, 0.06% $FeCl_3.6H_2O$ and 0.14% orcinol. These were heated with the specimen under investigation for 3 min. at 100°. Since then these conditions have been extensively modified.

The modifications described by Mejbaum (1939) estimate ribose at concentrations varying between 1-25µg/ml. utilising $FeCl_3$ as the catalyst, and the orcinol at a concentration of 10mg/ml. The sample is heated in the presence of an equal volume of reagent in a boiling-water bath for 20 min. If DNA is present the readings must be corrected because this substance is known to interfere, the error being as high as 12% (Webb and Levy 1958).

Drury (1948) employed ammonium ferric sulphate

as a catalyst, and Ceriotti (1955) has followed the example of Barrenscheen and Peham (1942) by utilising copper chloride ($CuCl_2$) as a catalyst.

The procedure outlined by Ceriotti (1955) consists of mixing equal volumes of the sample and the reagent and heating in a boiling-water bath for 40 min. After cooling, the coloured product caused by impurity, is extracted with iso-amyl alcohol, rendering the test less liable to interfering substances, as these are usually extracted by the alcohol. Furthermore, Ceriotti noted that the colour intensity remains constant or decreases only slightly after this period of heating. Lambert-Beer's law is obeyed at concentrations between 2-15 µg of ribose/ml.

Miller, Golder and Miller (1951) undertook an investigation of the orcinol reaction to determine the optimum conditions. They found that on increasing the concentration of orcinol, iron or HCl in the reagent, the colour intensity increased correspondingly until it reached a maximum, and ultimately any increases in the latter component resulted in a decreased colour intensity. Colour development is accelerated at high rather than low concentrations of orcinol and HCl, but it was relatively uninfluenced by the concentration of iron. When increasing the amounts of reagent, the blank reading is also augmented;

they, therefore, concluded that the conditions described by Drury, (1948) were those most closely approximating to the optimum.

As previously mentioned, the orcinol reaction is not completely specific and the following substances are known to interfere: hexoses, sucrose, high concentrations of protein, phosphates, TCA and DNA, (Hutchinson and Munro 1961). In an attempt to overcome this problem Deken-Grenson and Deken (1959) used Dowex-2 resin to remove impurities which reacted with orcinol.

During investigations on the estimation of RNA in rat liver, Fleck and Munro (1962) observed that after digestion for 1 hr. at 37^{o} in normal or 0.3N-KOH, all the RNA of the tissue was acid soluble and the orcinol reaction provided a reasonable measure of the RNA present. However, considerable amounts of protein were released with the RNA fraction after 24 hr. digestion, and the readings obtained with this fraction rose by 8%, which coincided with the release of protein into the acid-soluble fraction of the digest.

A second method for the determination of ribose is that described by Dische and Borenfreund (1957). This consists of mixing the sample under investigation with phloroglucinol in a strongly acid medium, and heating the

mixture in a boiling-water bath for 15 min. after which the extinction is measured at 552mμ. The tests were carried out in the presence of glucose which did not interfere with the reaction. The advantage of the method is its specificity especially in the presence of other sugars. However, the minimum concentration of ribose or its esters necessary is five times as high as in the orcinol reaction (Dische and Borenfreund 1957).

Webb (1955) developed a colourimetric procedure whereby the furfural formed by heating ribose in the presence of 4 N HCl can be extracted with Xylene. The xylene extract is then reacted with p-bromophenylhydrazine in HCl-ethanol solution, which produces a yellow colour with a maximum absorption at 450mμ. Under standardized conditions the yellow colour developed is a measure of the amount of RNA present, since DNA does not interfere. The xylene extraction minimizes the effect of interfering substances. Lambert-Beer's law is obeyed over the range 9.4-150μg/ml. of RNA. Galacturonic acid is known to interfere with the reaction.

The cysteine-sulphuric acid method for the determination of ribose as described by Dische (1949) is not specific, for most sugars give a colour with these reagents. The breakdown products produced from sugars

on hydrolysis with sulphuric acid when combined with cysteine at room temperature produce colours with absorption maxima varying between 375 - 410mμ depending on the type of sugar. Gurin and Hood (1941), by treating certain sugars with carbazole, were able to distinguish between xylose, aldopentoses and methyl pentoses. A modification was introduced by brominating the pyrimidine nucleotides. This afforded a means of determining the total pentoses present in the sample.

The anthrone reaction for the determination of Hexoses has been known for many years. This test has been successfully adapted for estimating pentoses in RNA and DNA. Bailey (1958) carried out investigations by treating anthrone with certain pentoses; D- and L- arabinose and D-ribose. The anthrone reagent, which is dissolved in strong sulphuric, is added to the sample and the contents are mixed while immersed in a refrigerant and then heated for 7 min. at 100°, cooled, and the absorption measured at 625mμ. Bailey found that the blue-green colour developed by these sugars was primarily dependant on the concentration of anthrone, an excess of which tended to destroy the colour rapidly.

Gary and Klausmeier (1954), using the same reagent, reduced the heating period to 2.5 min. prior to measuring the absorption at 265mμ. Unfortunately and not unexpectedly

hexoses interfere and must be removed. However, mixtures of nucleic acids can be determined in the presence of small quantities of each other.

As described earlier, the ultraviolet absorption spectrum of the purified antigen solution suggested that the antigen contained nucleic acid as the maximum was between 260 - 265mμ.

As the amount of antigen solution available for the biochemical tests was very small, this placed severe limitations on certain of the biochemical tests. Modifications were introduced to increase the sensitivity and decrease the volume of antigen required for the tests, and subsequently the orcinol reaction was found to be sensitive and appeared at least as specific as any of the others. The low reagent blank reading was an added advantage of this method.

The following section deals with the modifications introduced and the tests carried out on the soluble antigen for the quantitative and qualitative estimation of nucleic acids.

MATERIALS AND METHODS.

Chemicals. Cupric chloride ($CuCl_2.2H_2O$) Analar grade B.D.H.

Orcinol ($CH_3.C_6H_3(OH)_2.2H_2O$). Laboratory grade reagent B.D.H.

Hydrochloric acid. Analar grade B.D.H.

Sp. Gr. 1.18.

Ribonucleic acid (highly polymerized) prepared from yeast was obtained from B.D.H. Cape Town.

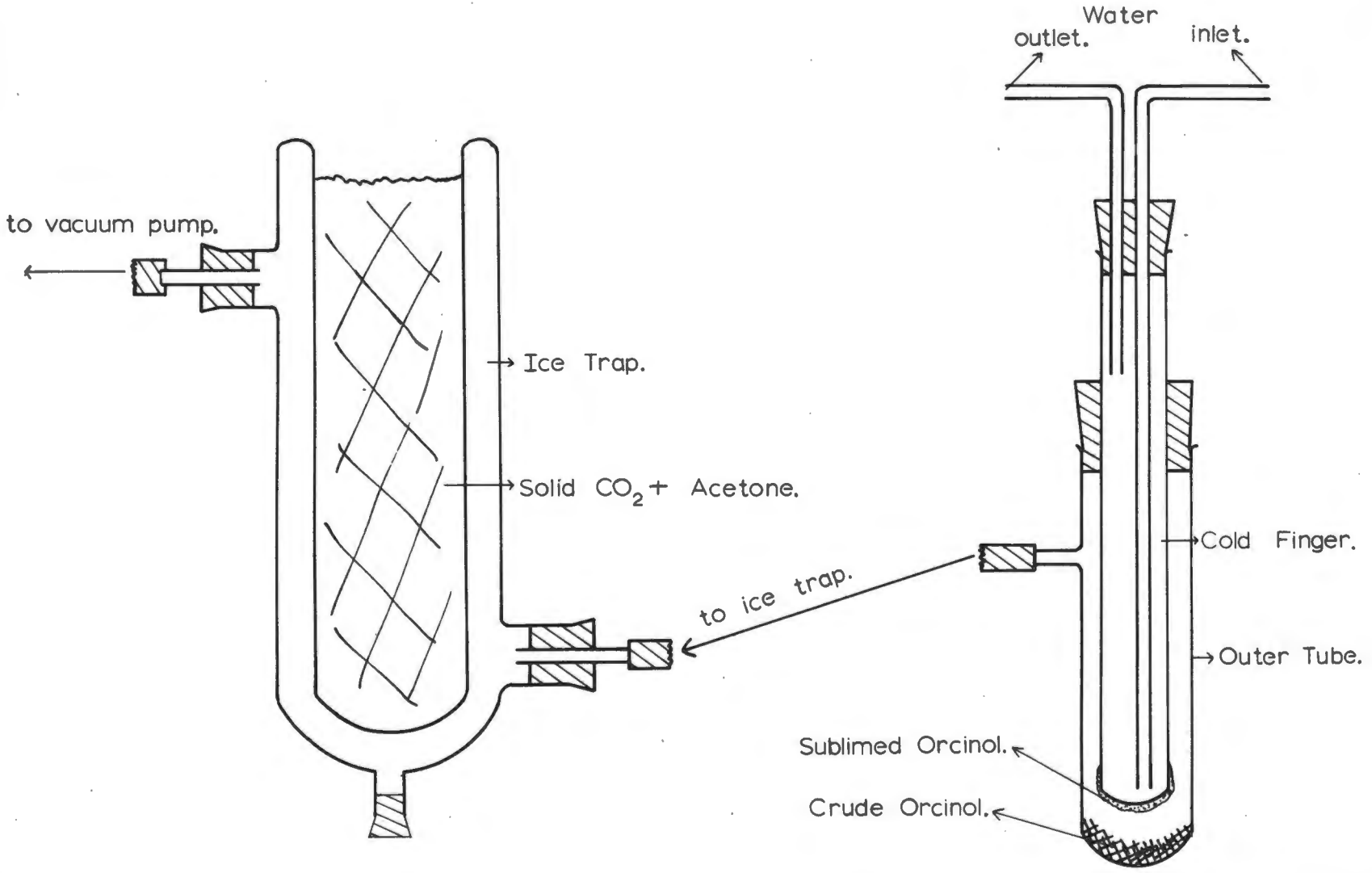
D(-) Ribose. Laboratory grade reagent B.D.H.

Orcinol, was purified according to the method of Katz, Larsson and Mead (1967). The commercial hydrated orcinol was first dehydrated by azeotropic distillation with benzene. Redistilled benzene (approximately 10ml./g.) was added to the crude commercial orcinol and distilled off over a boiling water bath. The initial distillate was cloudy, indicating the presence of water; but the last traces of benzene were clear. Any benzene remaining in the brown crystalline residue was evaporated in a current of air and the residue sublimed at 100° in a high vacuum apparatus shown in Fig. 7.

The dried orcinol was placed in the outer tube and a cold finger was extended within it to a depth of approximately 1 cm. above the residue. The apparatus was

Fig. 7.

Apparatus used to sublime the orcinol.


Water
outlet.
inlet.
to vacuum pump.
Ice Trap.
Solid CO_2 + Acetone.
to ice trap.
Cold Finger.
Outer Tube.
Sublimed Orcinol.
Crude Orcinol.

immersed in a boiling water-bath and the system evacuated through a dry-ice trap. The trap was used to prevent the carry over of moisture, and so prevent contamination of the oil in the "Speedivac" high vacuum pump (W.Edwards, London). A white sublimate collected at the base of the cold finger leaving a black residue at the base of the outer tube. The white crystals were removed, ground in a mortar and sublimed for a second time, M.pt. 106° (Lit.). The powder became discoloured during storage in the dark, but could be used for 3 - 4 weeks after sublimation without reducing the sensitivity of the test.

Spectrophotometry. Four glass micro cells (approximate volume 0.5 ml.) were marked a,b,c, and d, and each was filled with distilled water. Starting with glass cell (a) as the blank, the absorptions of the other three cells were measured against it at 670mμ. The procedure was repeated, each cell in turn being used as a blank. Greatly divergent readings were obtained; some cells showing an apparent extinction as high as 0.017. However cell (a) read against cell (c), when both were filled with water and placed in the carrier in the same positions, always gave a reading of zero. Throughout these investigations cell(a) was filled with water and cell (c) contained the solution or blank

under investigation. After a measurement had been made, cell (c) was emptied, washed and dried and refilled with the next sample.

Preparation of standards. As this method was used primarily for the estimation of ribose, this substance was used for the preparation of the standards. The primary ribose standard was prepared by dissolving 10mg. of ribose in 100ml. of distilled water, and a few drops of chloroform were added as a preservative. Further dilutions, prepared individually in water, contained 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 µg ribose/ml.

Preparation of reagent. A stock solution containing 0.004M-$CuCl.2H_2O$ in concentrated HCl was prepared and stored in the fume cupboard. Immediately before use, 200mg. of freshly sublimed orcinol were dissolved in 20ml. of concentrated HCl, 2.5 ml. of the $CuCl_2$-HCl solution were added and the volume was made up to 25 ml. with concentrated HCl. The reagent was clear, but before addition to the sample, it was filtered with gentle suction through a No 4 sintered glass filter which had previously been rinsed with concentrated NCl and two portions of the reagent.

Method for the micro-estimation of ribose. To 0.25 ml. of each standard solution, unknown and water (blank),

prepared in duplicate, was added an equal volume of freshly prepared and filtered reagent. Evaporation was prevented by sealing the test tubes (12 x 150 mm) 2-3 cm. from the mouth. Sealing was done with a Chance "flamemaster" supplied with coal gas, air and oxygen to give a roaring flame, in contrast to a long silent flame which was produced with an excess of oxygen (see experimental).

After sealing, the tubes were immersed in a boiling-water bath for 40 min. and then cooled by immersion in tap water for 2-3 min. The tubes were left to drain in a perpendicular position for 10-15 min. before opening, and the absorption measured in the glass microcells against water at 670mμ (see spectrophotometry). The solutions were not extracted with iso-amyl alcohol as suggested by Ceriotti (1955).

EXPERIMENTAL.

Initially the test was performed exactly as described by Ceriotti (1955), with two minor modifications. The volumes of the sample and reagent were reduced to 0.25 ml. and the samples were not extracted with iso-amyl alcohol.

The agreement between duplicate standards was poor, and on numerous occasions the standards and blanks were found to have a pinkish-brown discolouration after the 40 min. heating period. This poor agreement between duplicate samples was originally thought to be due to some imperfection in the glass cells used for measuring the absorption of the samples. This assumption was investigated (see spectrophotometry). Once this source of error had been eliminated, tests using standards of ribose were repeated, but although the agreement between duplicates had improved, the discolouration was still found periodically in some of the tubes. Unfortunately the discolouration absorbed maximally at 490mμ and to some extent at the same wavelength at which the samples were read i.e. 670mμ, and an experiment was done to prove this. A sample containing 0.25 ml. of water and an equal volume of reagent was prepared according to Ceriotti's (1955) original method, by dissolving 2 mg. of orcinol/ml. in the copper HCl reagent. The solution was well mixed and heated for 40 min. at 100°. The absorption was measured

against distilled water between 400 and 700mμ at 10mμ intervals in the spectrophotometer. The results are shown in Table 3 and graphically in Fig. 8. The solution consisting of water and reagent had a pinkish discolouration.

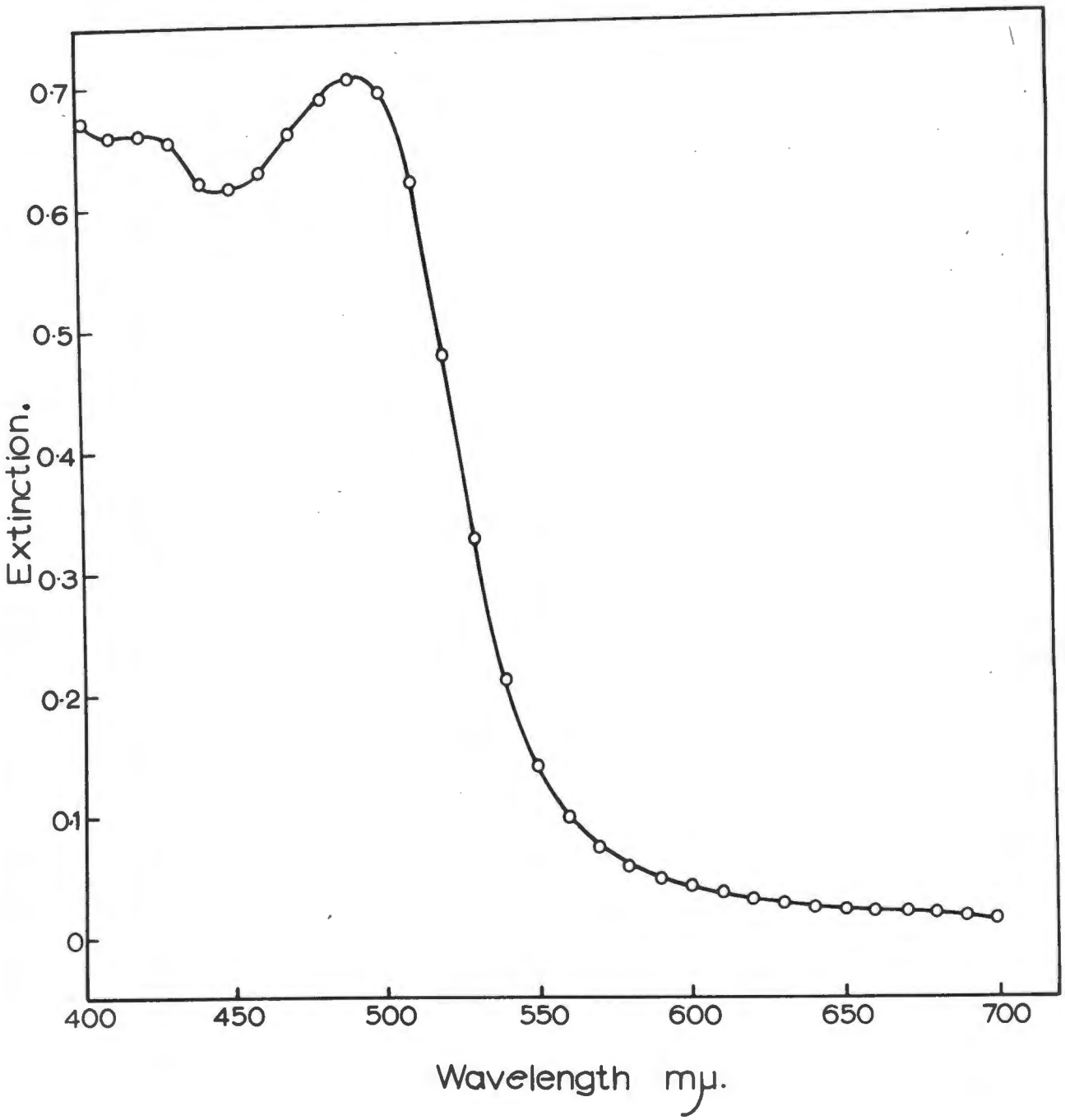
Table No 3.

Absorption measurements of a solution containing equal volumes of orcinol reagent and water after heating at 100° for 40 min.

Wavelength in mμ.	Absorption Mean of 2 readings.	Wavelength in mμ.	Absorption Mean of 2 readings.
400	0.670	560	0.100
410	0.658	570	0.075
420	0.660	580	0.061
430	0.652	590	0.051
440	0.620	600	0.045
450	0.615	610	0.040
460	0.628	620	0.034
470	0.660	630	0.030
480	0.690	640	0.028
490	0.705	650	0.026
500	0.690	660	0.025
510	0.622	670	0.024
520	0.480	680	0.022
530	0.328	690	0.018
540	0.212	700	0.016
550	0.140		

Fig. 8.

Absorption measurements of a solution containing equal volumes of water and orcinol reagent with the characteristic discolouration mentioned in the text. The tube was sealed and the colour developed at 100° for 40 min.


0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Extinction.
400
450
500
550
600
650
700
Wavelength mμ.

From Fig 8 the maximum absorption is at 490mμ, with a small but significant absorption at 670mμ, which had to be eliminated because the readings obtained for the lowest concentration of ribose, before correction by subtracting the blank value, closely approximated to the value obtained at 670mμ during this experiment. Furthermore, it must be appreciated that the sample under investigation contained no ribose. In all probability the extraction with iso-amyl alcohol would have removed the impurity, but this possibility was not investigated because the cause of the discolouration had not been found.

All the glassware used during this investigation was then washed with hot concentrated nitric acid, followed by numerous washings with distilled water in an attempt to eliminate the discolouration, which was thought at this stage to be due to imperfectly cleaned glassware. However, certain tubes were still discoloured.

The appearance of this discolouration was unpredictable. No discolouring was noticed on some days so it was decided to filter the reagent, prior to its addition to the samples. The results were more consistent, but the recurrence of the discolouration in certain tubes was still noticeable.

The orcinol used for the preparation of the reagent

at this stage of the investigation was not as yet purified as described under methods. Consequently an experiment was designed whereby the reagent was prepared with freshly sublimed orcinol (see methods), and in conjunction with this experiment the optimum concentration of orcinol was investigated.

Four reagents were prepared containing respectively 2, 5, 8 and 12 mg. orcinol/ml. A blank and two ribose standards (1.6 and 3.2 µg. ribose/ml.) were prepared with each reagent, and after mixing in equal quantities the solutions were heated at 100° for 40 min. The colour of the freshly sublimed orcinol was white in contrast to the orange-brown colour of the orcinol obtained from the manufacturer. The results are given in Table 4 and graphically in Fig. 9.

Table No. 4.

Absorption measurements of blanks and ribose standards treated with different concentrations of orcinol reagent.

Orcinol conc. in mg/ml.	Ribose conc. in µg/ml.	Absorption readings at 670mµ.	Corrected absorption reading. (minus blank)
2	Blank	0.022	
	1.6	0.115	0.093
	3.2	0.174	0.152
5	Blank	0.029	
	1.6	0.138	0.109
	3.2	0.257	0.228
8	Blank	0.024	
	1.6	0.154	0.130
	3.2	0.269	0.245
12	Blank	0.0375	
	1.6	0.164	0.1265
	3.2	0.271	0.2335

As the concentration of orcinol was increased the sensitivity of the reaction was increased, but at a concentration of 12 mg./ml. the sensitivity decreased as the blank reading was considerably augmented. The optimum concentration of orcinol was, therefore, taken to be 8 mg./ml. However, certain tubes still showed the discolouration, which was obviously not due to the orcinol or its state of purity.

Inconsistent results could be correlated with the brown discolouration present in certain tubes. This colour would not have been observed if the iron catalyst had been used in place of the copper, as the copper reagent was found to be almost colourless after its addition to the sample.

Ultimately it was noticed that the occurrence of the discolouration was due to the type of flame used for sealing the tubes. Tubes sealed 2 -3 cm. from the mouth in a flame produced from a mixture of coal gas, air and oxygen ("roaring flame") gave no discolouration when the colour was developed. Tubes sealed, however, in a flame produced by a mixture of coal gas, air and an excess of oxygen (silent flame) developed the typical discoloura- which lead to the disagreement between duplicate samples. As both types of flame had at first been used during the

sealing process it was understandable that the discolouration was noticed intermittently.

An experiment was done to verify this assumption. Standards were prepared in duplicate, and series A (see Table 5) were sealed in a "roaring flame" and series B in a "silent flame". The concentration of orcinol was 8 mg./ml. in the copper-HCl reagent, and an equal volume of this reagent was added to each solution before the tubes were sealed and heated. The reagent was filtered before addition to the solutions. The results are shown in Table 5 and graphically in Fig. 10.

Table No. 5.

Absorption measurements of blanks and ribose standards sealed in a "roaring" and "silent" flame.

	SERIES A Roaring flame		SERIES B Silent Flame.	
Ribose conc. µg./ml.	Absorption at 670 mµ.	Absorption corrected nett.	Absorption at 670 mµ.	Absorption corrected nett.
Blank	0.013	0	0.030	0
0.1	0.020	0.007	0.0395	0.0095
0.2	0.028	0.015	0.057	0.027
0.4	0.042	0.029	0.0695	0.0395
0.8	0.069	0.056	0.089	0.059
1.6	0.125	0.112	0.140	0.110
3.2	0.240	0.227	0.299	0.269

Fig. 9.

The effect of increasing concentration of freshly sublimed orcinol on the sensitivity of the test. Blanks and two standards (1.6 and 3.2 µg. ribose/ml.) were mixed with an equal volume of reagent prepared by dissolving 2mg. orcinol/ml., (▲——▲), 5mg. orcinol/ml., (○——○), 8mg. orcinol/ml. (●——●), and 12mg. orcinol/ml. (△——△) in copper-HCl solution.

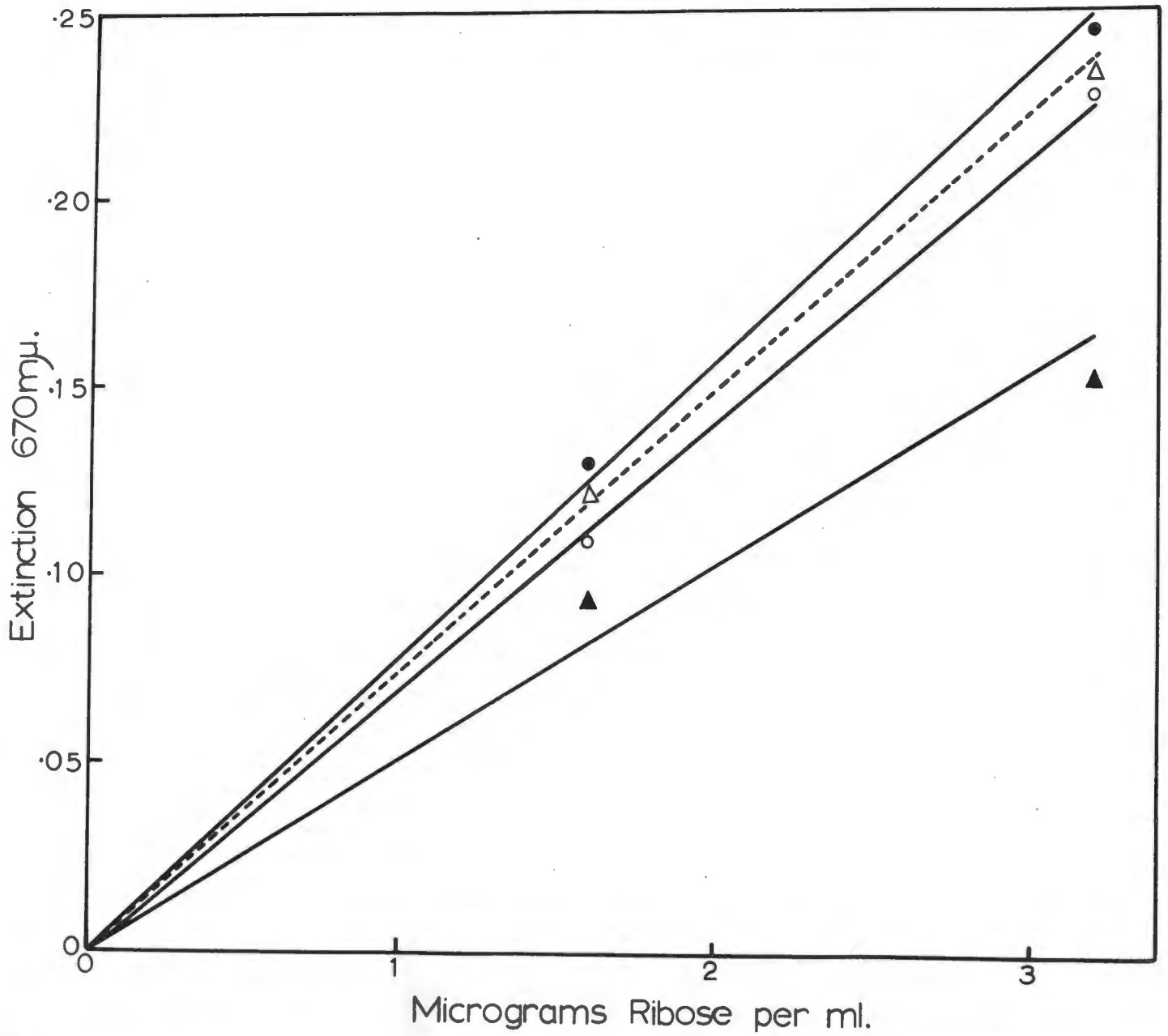
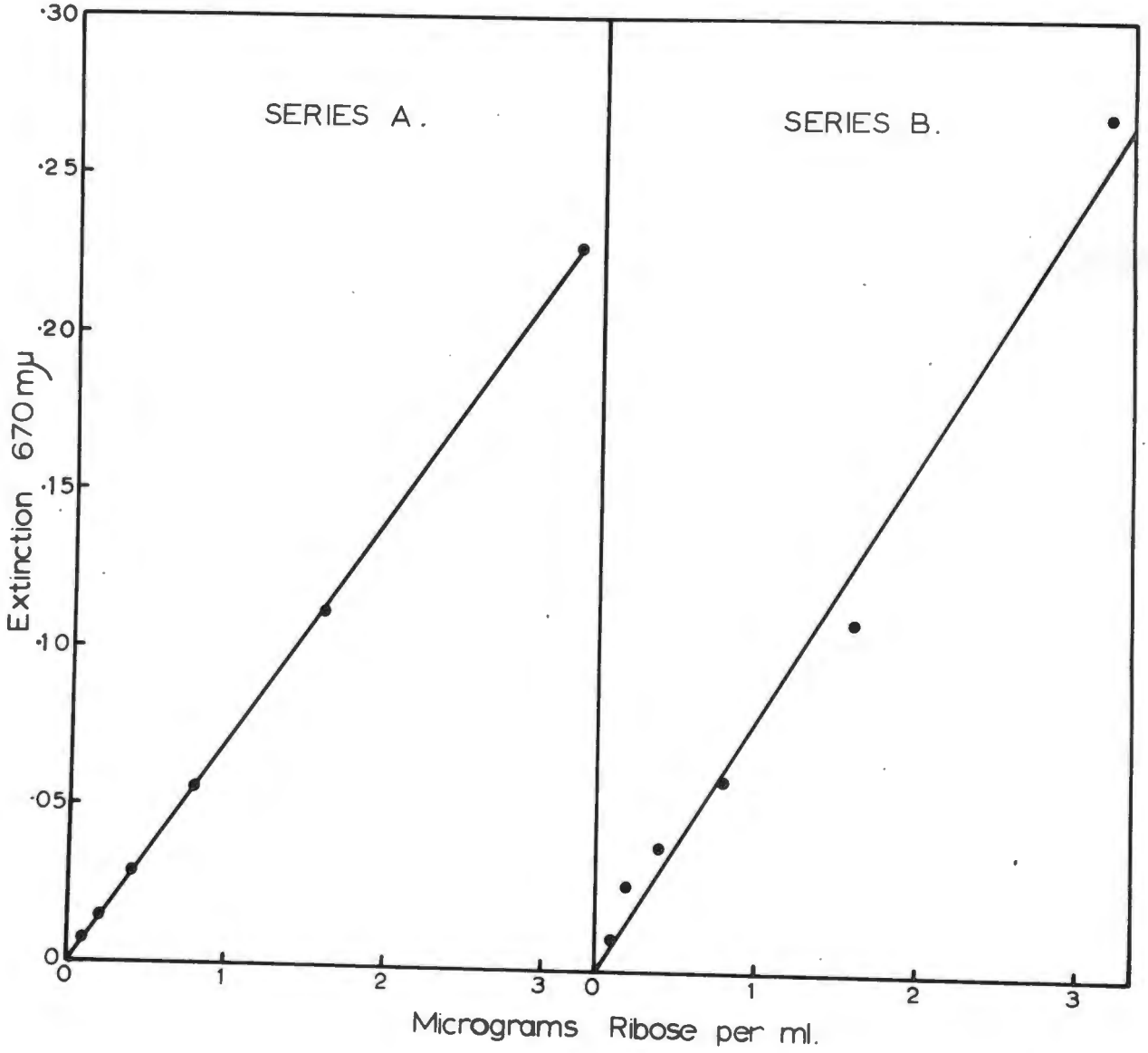

·25
·20
·15
·10
·05
0
0
1
2
3
Extinction 670mμ.
Micrograms Ribose per ml.

Fig. 10.

The effect of sealing tubes in a "roaring" and "silent" flame. Blanks and standards (0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µg. ribose/ml.) were prepared in duplicate and mixed with an equal volume of reagent and half the tubes (series A) were sealed in a roaring flame, and series B were sealed in a silent flame.


·30
·25
·20
·15
·10
·05
0
Extinction 670 mμ
SERIES A.
SERIES B.
0
1
2
3
0
1
2
3
Micrograms Ribose per ml.

The discolouration present in certain tubes sealed in a "silent flame" was due to some product of combustion. Numerous substances could have been responsible for the discolouration, but the possibility of formaldehyde, which is a known by-product of incomplete combustion of coal gas, as the substance responsible for the discolouration, was investigated.

A 40% ($^{W}/_{V}$) solution of commercial formalin was diluted with water to give solutions containing 400, 40, 4 and 0.4 µg. formalin/ml. To 0.25 ml. of these dilutions was added an equal volume of reagent and the colour developed as before. The results are shown in Table 6.

Table No. 6.

The effect of various concentrations of formalin on the orcinol reaction.

Formalin conc. µg./ml.	Absorption at 670mµ.	Absorption at 670mµ (nett).	Absorption at 490mµ.
Blank	0.012	0	0.0245
400	Precipitated	Precipitated	Precipitated
40	0.012	0	0.570
4	0.012	0	0.187
0.4	0.012	0	0.042

The solution containing 400 µg. formalin/ml. caused precipi-

tation of the contents of the tube. All the remaining tubes gave a distinct orange colour, which was not similar to that seen during the ribose determinations. Furthermore the discolouration did not absorb at 670mμ as the readings obtained for all the solutions including the blank were identical. However, the absorption at 490mμ was high.

The desirability of filtering the reagent was once again investigated and two series of tubes were prepared, containing standards and reagent which were sealed and heated in the usual manner. Series A contained unfiltered reagent whereas that used in Series B had been filtered as described above. The results are recorded in Table 7 and graphically in Fig. 11.

Fig. 11.

The effect of filtered and unfiltered orcinol reagent on blanks and standard ribose solutions. Blanks and standards were prepared in duplicate and an equal volume of unfiltered reagent added to the tubes in series A, and an equal volume of filtered reagent was added to the tubes in series B.

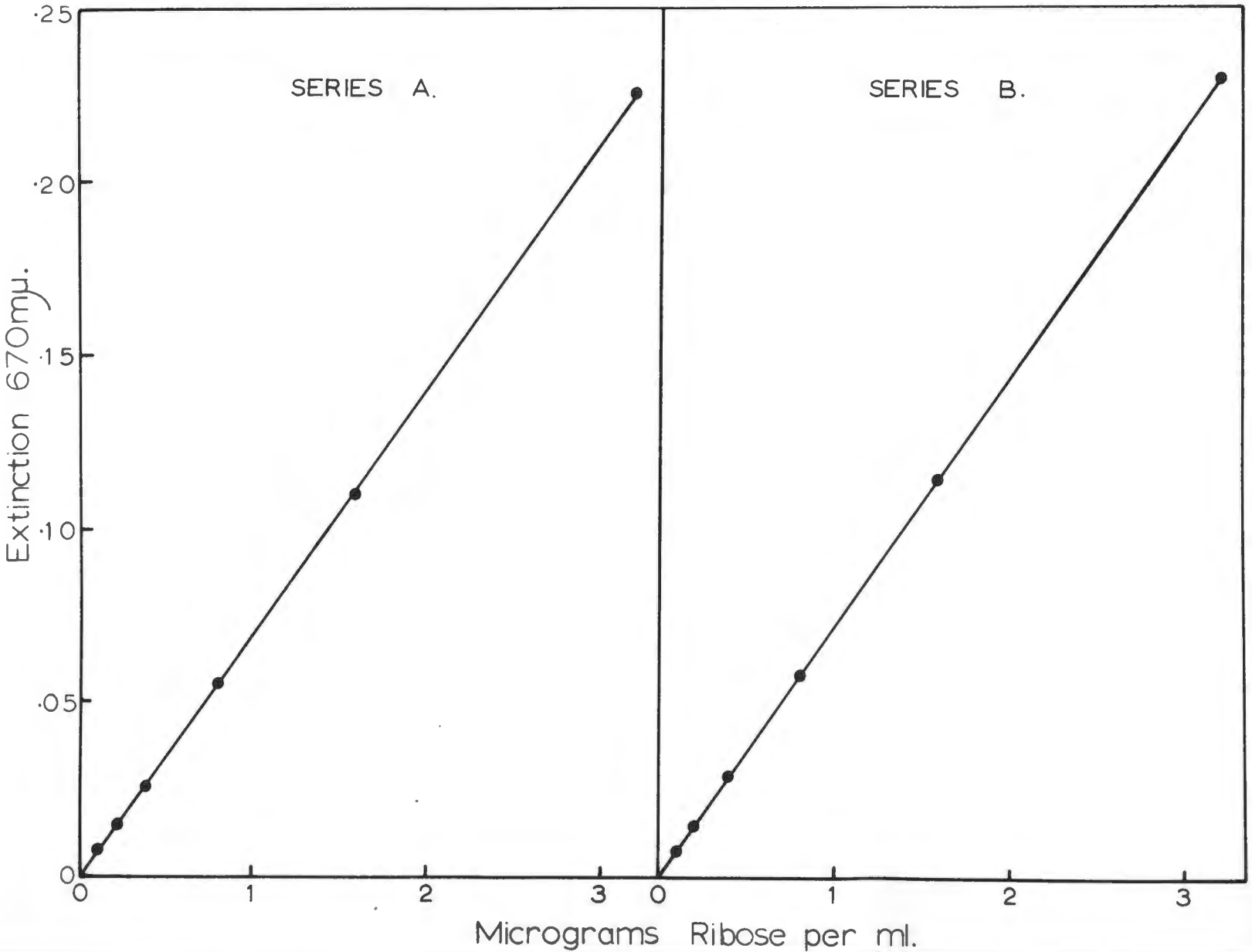

SERIES A.
SERIES B.
·25
·20
·15
·10
·05
0
Extinction 670mμ.
0
1
2
3
0
1
2
3
Micrograms Ribose per ml.

Table No. 7.

The effect of filtered and unfiltered orcinol reagent on blanks and standard ribose solutions.

	Series A Unfiltered reagent		Series B Filtered reagent	
Ribose conc. in µg/ml.	Absorption at 670mµ.	Absorption at 670mµnett	Absorption at 670mµ.	Absorption at 670mµnett.
Blank	0.017	0	0.017	0
0.1	0.025	0.008	0.025	0.008
0.2	0.033	0.016	0.033	0.016
0.4	0.0435	0.0265	0.047	0.030
0.8	0.0725	0.0555	0.0745	0.0575
1.6	0.1275	0.1105	0.132	0.115
3.2	0.2425	0.2255	0.247	0.230

From the results it seemed irrelevant whether the reagent was used filtered or unfiltered and in all probability, therefore, it could have been omitted.

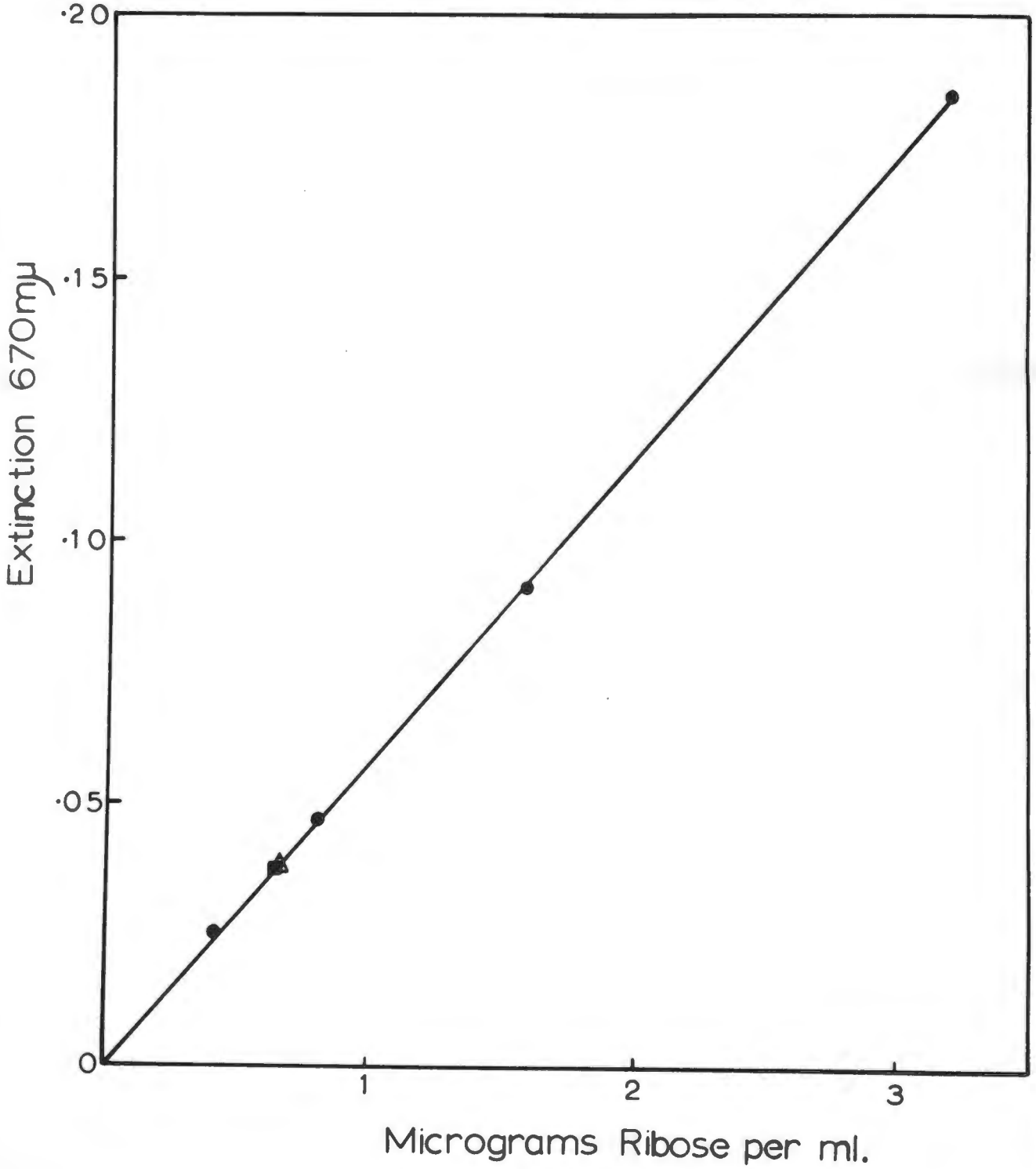
It was noted that 5 - 6 weeks after subliming the orcinol, the originally white product developed a pinkish-brown discolouration at the surface of the powder even though the container had been tightly stoppered and stored in the dark. It seemed that the material was once again reverting to its original state, probably due to oxidation.

Although the orcinol was discoloured it continued to give reproducible results when used in the preparation of the reagent. The discoloured orcinol was, therefore, used in numerous experiments without ill effect except that the sensitivity of the reaction was greatly reduced, but the blank reading remained constant. The experiment to be described proves these assumptions.

In conjunction with the above investigation the effect of protein on the colour development of a standard solution of RNA was investigated. A RNA solution was prepared by dissolving 5 mg. in 50 ml. of distilled water. (100 µg. RNA/ml.). Human serum albumen (HSA) (freeze-dried powder) 50 mg. was dissolved in 50 ml. distilled water to give a solution containing 1000 µg HSA/ml. The orcinol reagent was prepared by dissolving 8 mg. of the "aged" orcinol in each millilitre of the copper-HCl solution which was of the same age as the orcinol, for it had been prepared on the day that the orcinol was sublimed. Two solutions were prepared for investigation: (a) 0.5 ml. of the above RNA solution was diluted to 50 ml. with distilled water (1 µg./ml.) and (b) 0.5 ml. of the RNA solution was mixed with 9 ml. of the HSA solution and diluted to 50 ml. with distilled water, giving a concentration of 1 µg. of RNA/ml. and 180 µg /ml. of HSA. Tubes were set up containing 0.25ml.

Fig. 12.

The effect of "aged" orcinol and protein on the sensitivity of the orcinol test. A blank and four standards (●——●) and two solutions containing respectively 1µg. RNA/ml. (■) and 1µg. RNA + 180µg. HSA/ml. (Δ) were mixed with an equal volume of reagent prepared from "aged" orcinol.


·20
·15
·10
·05
0
Extinction 670mμ
1
2
3
Micrograms Ribose per ml.

of each of the above solutions and 0.25ml. was added to each sample of the reagent including the blanks and ribose standards, and the colour developed. The results are recorded in Table 8 and graphically in Fig. 12.

Table No. 8.

The effect of "aged" orcinol and protein on the sensitivity of the orcinol reaction.

Ribose conc. in µg./ml.	Absorption at 670mµ.	Absorption at 670mµ nett.
Blank	0.015	0
0.4	0.042	0.026
0.8	0.062	0.047
1.6	0.107	0.092
3.2	0.200	0.185
1µg. RNA/ ml.	0.0385	0.0235
1µg. RNA/ ml. and 180µg. of HSA/ml.	0.040	0.025

From the slope of the calibration curve it seemed that the sensitivity of the method had been reduced by about a third of its original value when freshly sublimed orcinol was used.

Secondly the results indicate that the orcinol should be freshly sublimed in order to obtain the most

sensitive result, although the "aged" orcinol did not interfere with the reaction and the blank reading was not augmented.

Finally, the presence of protein (HSA) at a concentration 180 times that of the RNA, only increased the reading by 6%.

The above experiment was repeated using a freshly prepared copper-HCl reagent, for it was thought possible that this solution might have been responsible for the loss of sensitivity, although it was unlikely-it contained no chemicals likely to break down on storage. The values obtained for duplicate standard solutions were in good agreement with those obtained using the old copper-HCl reagent, showing that the orcinol alone was responsible for the drop in sensitivity.

During the preparation of the standard stock solutions of ribose and RNA a few drops of chloroform were added as a preservative, and it was necessary to investigate the effect of this substance on the test. Samples of ribose and RNA solutions were prepared in duplicate. One tube was prepared in the presence of chloroform and the other without the presence of chloroform. Each was mixed with the reagent and the colour developed. The agreement between duplicates containing chloroform and no chloroform was good, indicating that

the presence of the chloroform did not interfere with the reaction.

During the purification of the soluble antigen one of the techniques used was exclusion chromatography on agarose pearls. The effluents obtained from these columns gave a positive test with the anthrone reagent (Katz, Larsson and Mead 1967), and it was therefore considered essential to determine whether a similar result would be obtained with the orcinol reaction. A column containing agarose pearls was fed with buffered saline A and allowed to flow freely until approximately 150 ml. of effluent had been collected. This was discarded and the next 50 ml. eluted was collected immediately afterwards and then dialysed against several changes of distilled water at 4^{o} to free it of the salts present in the buffered saline A. A second sample of buffered saline A (50 ml.) which had not passed through the column was dialysed concurrently with the other sample. Three solutions were prepared (a) by mixing 0.5 ml. dialysed effluent with 0.5 ml. of a ribose solution containing 0.4 µg/ml. Solution (b) was prepared by mixing equal volumes of dialysed effluent and distilled water, and solution (c) consisted of dialysed buffered saline A. Blanks and standards of ribose including the three above solutions were mixed with an equal volume of reagent and the colour

developed. The orcinol was not freshly sublimed for the preparation of the reagent in this experiment. The results are recorded in Table 9.

Table No. 9.

The effect of effluents from an agarose pearl column on the orcinol reaction.

Ribose conc. in µg./ml.	Absorption at 670mµ.	Absorption at 670mµ. nett.
Blank	0.009	0
0.2	0.021	0.012
0.4	0.031	0.022
0.8	0.052	0.043
1.6	0.096	0.087
Solution A	0.032	Soln.A minus Soln.B
Solution B	0.020	(0.012)
Solution C	0.019	

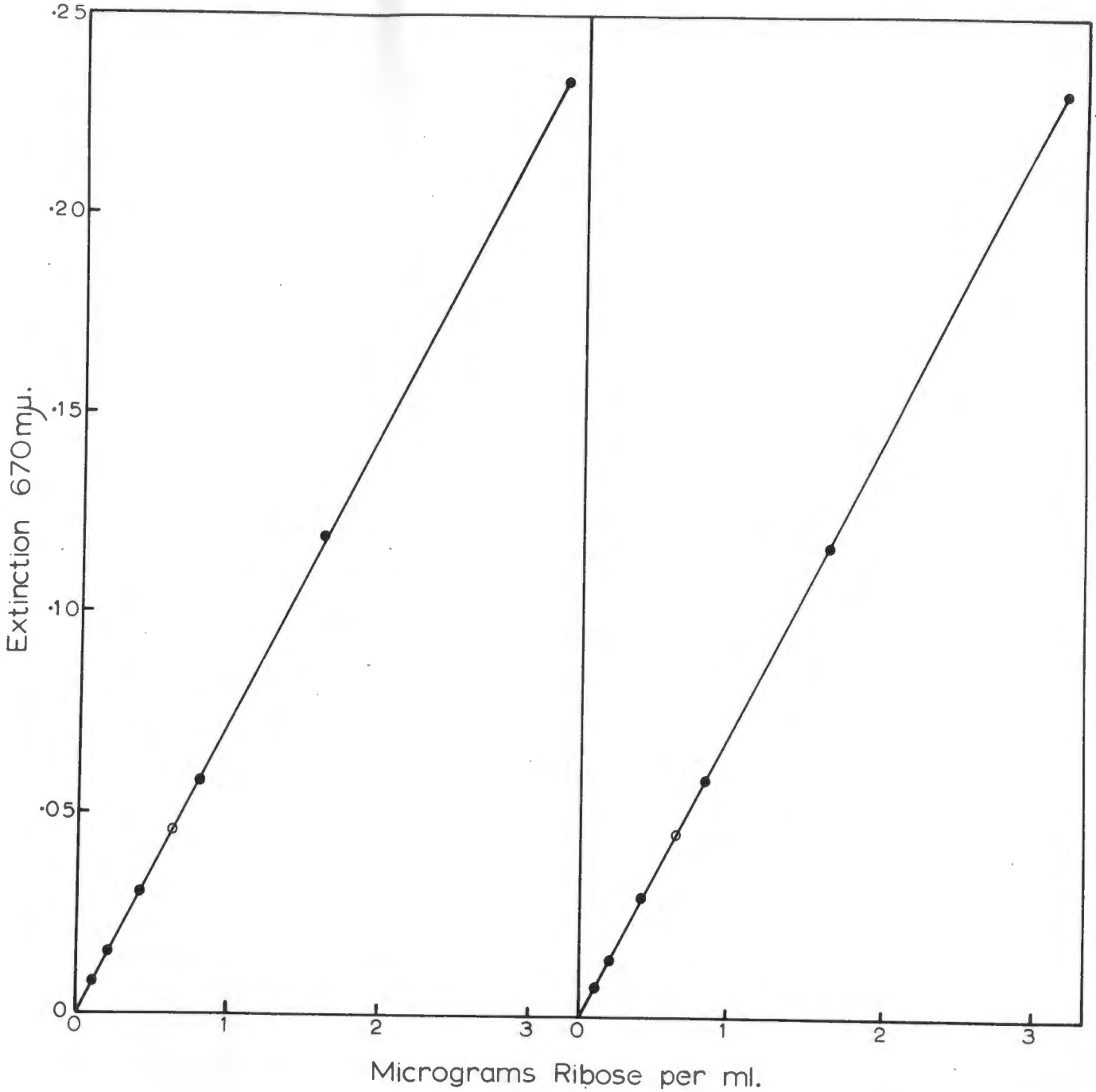
The agreement between solutions B and C indicate that no ribose was contributed by the agarose, although some unknown component of buffered Saline A or extractive from the dialysis casing had an appreciable apparent ribose content. Secondly, the sensitivity of the reaction was not affected by any extractive from the agarose or dialysis casing, because the difference between the extinctions of solution A and B corresponded exactly to the former's

content of 0.2 µg ribose/ml.

As the modified orcinol reaction continued to give accurate and reproducible results, it was applied to the purified solution of the soluble antigen. Blanks and standard ribose solutions were prepared. To conserve the antigen solution only 0.1 ml. was used and was diluted with 0.15 ml. distilled water. The blanks and standards were prepared in duplicate but not the antigen solution. The reagent was prepared from freshly sublimed orcinol at a concentration of 8 mg./ml. and the test performed as described in methods. The results are recorded in Table 10 and graphically in Fig. 13.

Fig. 13.

The first estimation of ribose in the solution of purified soluble antigen. Extinction at 670mμ (corrected for blank) of duplicate mixtures prepared with standard ribose solutions (●——●). Corrected extinction at 670mμ of a single reaction mixture prepared with 0.10 ml. of antigen solution and 0.15ml. of water (o).


·25
·20
·15
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·05
0
Extinction 670mμ.
0
1
2
3
0
1
2
3
Micrograms Ribose per ml.

Table No. 10.

The absorption measurements of blanks, ribose standards and the purified solution of the soluble antigen on treatment with orcinol.

Ribose conc. in μg./ml.	Absorption at 670mμ.	Absorption at 670mμnett.	Absorption at 670mμ.	Absorption at 670mμnett.
Blank	0.015	0	0.015	0
0.1	0.022	0.007	0.0225	0.0075
0.2	0.030	0.015	0.029	0.014
0.4	0.045	0.030	0.045	0.030
0.8	0.0735	0.0585	0.074	0.059
1.6	0.134	0.119	0.132	0.117
3.2	0.248	0.233	0.246	0.231
0.1 ml.antigen + 0.15 ml. H_2O	0.061	0.046		

The experiment described above was repeated the following day with a mixture of 0.15 ml. antigen and 0.1 ml. water. The same standards and blanks used on the previous day were used in this experiment, but fresh reagent was prepared. The results are recorded in Table 11 and Fig. 14.

Fig. 14.

The second estimation of ribose in the solution of purified soluble antigen. Extinction at 670mμ (corrected for blank) of duplicate mixtures prepared with standard ribose solutions (●——●). Corrected extinction at 670mμ of a single reaction mixture prepared with 0.15ml. of antigen solution and 0.1ml. of water (O).

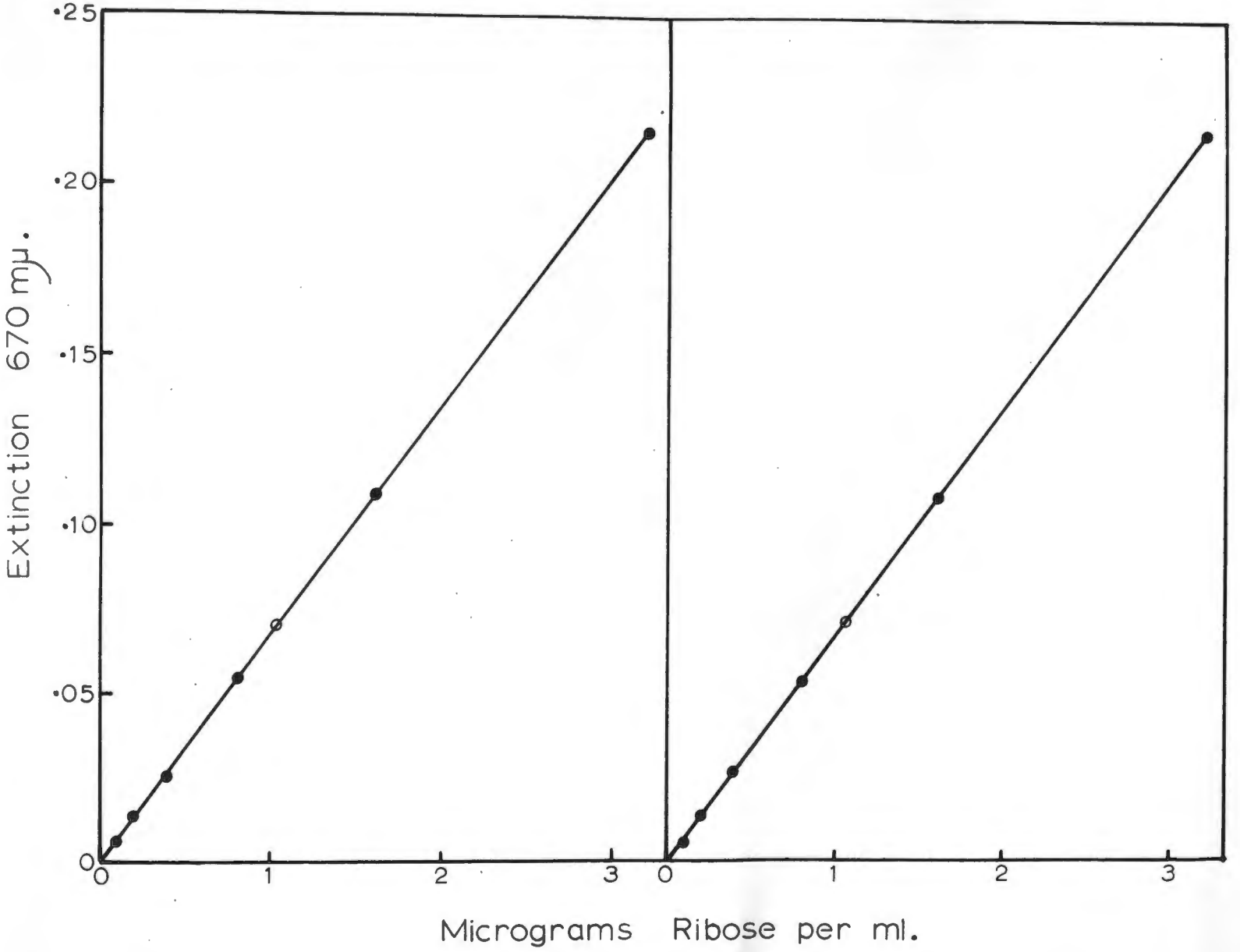

·25
·20
·15
·10
·05
0
Extinction 670 mμ.
0
1
2
3
0
1
2
3
Micrograms Ribose per ml.

Table No. 11.

The absorption measurements of blanks, ribose standards and a second sample of the purified solution of the antigen on treatment with orcinol.

Ribose conc. in µg./ml.	Absorption at 670mµ.	Absorption at 670mµ.nett	Absorption at 670mµ.	Absorption at 670mµnett.
Blank	0.014	0	0.014	0
0.1	0.019	0.005	0.020	0.006
0.2	0.028	0.014	0.028	0.014
0.4	0.040	0.026	0.041	0.027
0.8	0.069	0.055	0.068	0.054
1.6	0.1235	0.1095	0.122	0.108
3.2	0.230	0.216	0.2295	0.2155
0.15ml.antigen + 0.1 ml. H_2O.	0.086	0.072		

The ribose concentration of the two undiluted antigen solutions corresponded to 1.6 and 1.8 µg.ribose/ml. respectively. Assuming that only the purine-bound ribose was responsible for the colour developed and that the molecular ratio of purine to pyrimidine in the antigen RNA is 1:1, the antigen solution contains about 7.3 µg./ml. of RNA.

Discussion. Of the reagents available for the determination of ribose, orcinol was selected because of its sensitivity,

reasonable specificity and wide adoption in other laboratories. Of the catalysts available, copper was chosen rather than iron because it had given good results in the hands of Ceriotti (1955), and because moreover it provided a practically colourless reagent.

The method had to be adapted for use with small volumes and dilute solutions, and it appeared essential to avoid any losses by evaporation, by carrying out the heating in sealed tubes. Apart from using very clean glassware, it was found that precautions were necessary in sealing the tubes and in the spectrophotometric examination.

The sensitivity of the reaction was increased by approximately one third of its original value by using freshly sublimed orcinol, and also by increasing the concentration of this material four fold. Unexpectedly, this increment in the concentration of the orcinol did not augment the blank reading which would have been anticipated for it was very sensitive to any changes in its composition.

The ageing of the orcinol with the accompanying discolouration reduced the sensitivity without increasing the blank reading. Protein as far as could be judged had little if any effect at relatively high concentrations. Similarly chloroform at concentrations necessary for

preserving the ribose standards had no effect on the test.

The ribose estimations were done in a region well below the normal limits of the test (Ceriotti 1955), with standards containing as little as 0.025µg. ribose/ 0.25 ml. and a maximum of 0.8µg. ribose/0.25 ml., which were used for establishing the calibration curve.

Two tests with the antigen solution indicated an apparent content of 1.6 and 1.8µg. ribose/ml. Assuming that only the purine-bound ribose was responsible for the colour developed, and that the molecular ratio of purine to pyrimidine nucleotides in the antigen RNA was 1:1, the antigen solution contained about 7.3µg./ ml. of RNA.

CHAPTER SEVEN.

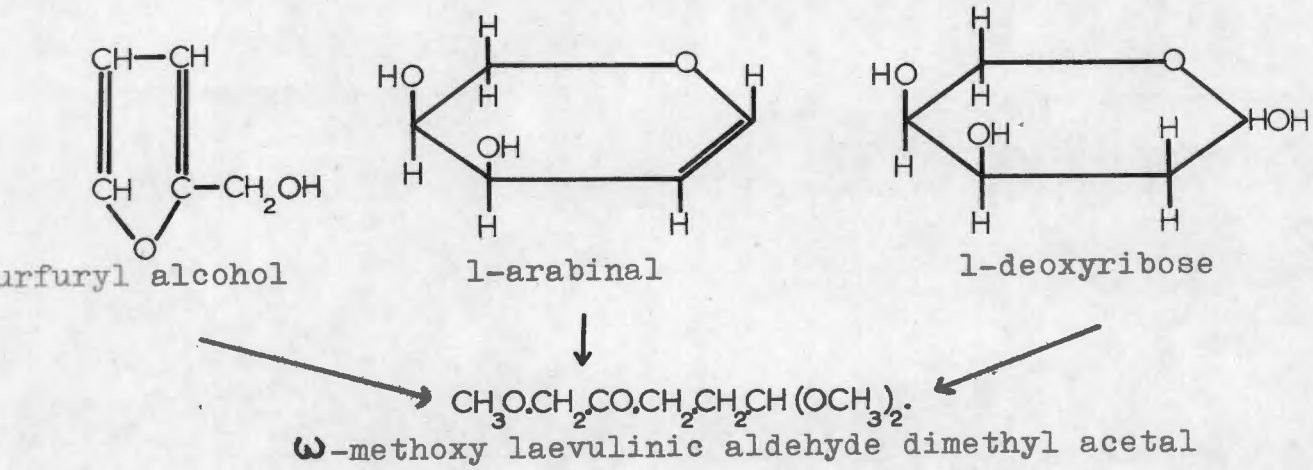
COLOURIMETRIC METHODS FOR THE ESTIMATION OF DEOXYRIBOSE.

Introduction. The techniques for estimating nucleic acids in general have been discussed in the previous chapter, and only those capable of distinguishing DNA will be dealt with in this section. The colourimetric estimations of DNA include reactions with diphenylamine, cysteine, indole, tryptophan, phloroglucinol, carbazole, p-nitrophenylhydrazine or anthrone. A method frequently used is the diphenylamine reaction of Dische, of which there are numerous modifications (Hutchinson and Munro, 1961).

The mechanism of the diphenylamine reaction has been investigated by Stacey, Deriaz, Teece and Wiggins (1946). They found that three substances, furfuryl alcohol, l-arabinal and l-deoxyribose, could be converted by special treatment to ω-methoxy laevulinic aldehyde, and they proved that it was this labile aldehyde which then treated with diphenylamine was responsible for the development of the blue complex.

Furthermore, they pointed out that sucrose and fructose readily yield ω-hydroxymethyl furfural with acids, and if heated under slightly increased acid concentrations

in the presence of diphenylamine a blue colour identical with that given by ω -methoxy laevulinic aldehyde dimethyl acetal was obtained. Therefore the reaction is not specific for deoxyribose and precautions must be taken to avoid unspecific colour reactions.



ω-methoxy laevulinic aldehyde dimethyl acetal

Hydrolytic degradation of DNA under the conditions used in its estimation, as in the case of RNA, leads to the liberation of free deoxyribose when linked to purine nucleotides. Brady and McEvoy-Bowe (1951) have claimed complete reaction from pyrimidine-bound ribose after first brominating these nucleotides.

The diphenylamine reaction was originally carried out in a mixture of glacial acetic acid and sulphuric acids at 100° for 10 min. Burton (1956) found that the reaction could be made 3.5 times more sensitive

by the addition of acetaldehyde to the diphenylamine reagent, and by carrying out the test at a lower temperature (30°) and over a longer period of time (16 - 20 hr.). The method is less susceptible to interference, but cysteine and ascorbic acid reduce the colour development.

Nigon and Daillie (1958) have modified the reagent by increasing the concentration of diphenylamine to 2%. They were able to determine the DNA present in a single larva of Drosophila, by reducing the method to an ultramicro scale.

A second method described by Dische (1929) is the determination of deoxyribose, using indole. Numerous modifications of the method exist, one commonly quoted being that of Ceriotti (1952). The sample of DNA is treated with indole in the presence of HCl, by heating for 10 min. at 100°. When cool the sample is extracted with chloroform which removes any yellow or red pigments formed by interfering substances, but leaves the yellow pigment produced by deoxyribose in the aqueous phase.

Small amounts of RNA or ribose and several other carbohydrates give colours of varying intensity with indole, but these colours are removed by the chloroform extraction. Ceriotti (1952) found that arabinose yielded a colour which was not fully extractable

with chloroform, and that TCA, but not PCA extracts of tissues, inhibited the colour development.

A micromethod devised by Bonting and Jones (1957) determines DNA at concentrations between 0.2 and 2.0 µg./50µl. The solution is extracted once only with 10 volumes of chloroform.

A second micromethod described by Keck (1956) permits the determination of 0.1-1.0µg DNA/20µl. The samples are extracted twice with equal volumes of amyl acetate which completely remove the colour due to arabinose. TCA was found not to interfere with the reaction. On applying the indole and diphenylamine methods to perchloric acid extracts of DNA fractions prepared from plant, animal and bacterial sources, Deken-Grenson and Deken (1959) showed good agreement between the two methods.

The cysteine-sulphuric acid reaction described by Dische (1949) has also been applied to the determination of deoxyribose at concentrations varying between 50-500µg. DNA/ml. Furthermore, this author has pointed out that protein does not interfere with the reaction.

Webb and Levy (1955) have developed a sensitive method for the estimation of DNA at concentrations between 10-300µg./ml. The hydrolysed sample is treated with p-nitrophenylhydrazine reagent in a boiling-water bath

for 20 min. After cooling, the solution is extracted with n-butyl acetate to remove interfering substances and the remaining aqueous layer is mixed with NaOH to develop the colour, which is measured at 560mμ. The p-nitrophenylhydrazine reaction is specific for deoxyribose under the conditions of the test. The authors claim a greater sensitivity for this method than is possible with diphenylamine.

A highly sensitive and specific fluorimetric procedure for determining deoxyribose has been developed by Kissane and Robins (1958). The procedure is based on the measurement of the fluorescent product of the reaction between 3,5-diaminobenzoic acid and deoxyribose. The procedure, which is specific for DNA as opposed to RNA, permits the measurement of as little as 0.002μg of DNA in a final volume of 50μl. The presence of substances other than that being determined does not seem to interfere in the measurement of the fluorescence.

The reaction of Tryptophan with deoxyribose in the presence of perchloric acid is a rather insensitive method for the determination of DNA (Cohn 1944). The red colour which develops during the reaction is extracted into iso-amyl alcohol, to separate it from the spurious colours which develop due to the presence of protein or other

interfering substances. The method is not specific for deoxyribose.

An extremely sensitive method was developed for the determination of 2-deoxysugars by Waravdekar and Saslaw (1957). It depends upon the oxidation brought about by periodate and the estimation of the resulting malonaldehyde with 2-thiobarbituric acid.

The anthrone reaction (Gary and Klausmeier 1954), is carried out by mixing the sample under investigation with the reagent and heating for 10 min. in a boiling-water bath, in contrast to that of RNA which was heated for 2.5 min. The colour developed, which is red in contrast to that of RNA which is blue, is read at 550-560 mμ whereas that of RNA is measured between 620-625 mμ. Mixtures of nucleic acids may, therefore, be analysed. However, the method is not specific for RNA and DNA, for most carbohydrates give a colour with this reagent.

MATERIALS AND METHODS.

Introduction. As the amount of purified soluble antigen available for the determination of deoxyribose was limited, the test had to be modified to the micro scale. Three methods were investigated: (a) the diphenylamine reaction according to Burton (1956), (b) the indole reaction according to Keck (1956), and (c) the indole reaction according to Ceriotti (1952). The procedures described by Burton and Ceriotti were found to be the most suitable, but unfortunately there was not sufficient antigen available to apply Ceriotti's method. The method according to Burton (1956) is described below, and an attempt has been made to adapt this method to a micro scale.

Materials.

0.005N-NaOH (approximate) was prepared by dissolving 0.2g NaOH (Analar-BDH) in 1 litre of distilled water.

Glacial acetic acid (Merck- "pro analysi") was redistilled.

Sulphuric acid (MAR grade BDH) 98%.

Perchloric acid (Merck "pro analysi") 60%.

Acetaldehyde (CH_3CHO BDH) was redistilled and an aqueous solution of CH_3CHO prepared which contained 16mg. CH_3CHO/ml.

Diphenylamine (Analar BDH) was purified by steam distillation.

Deoxyribonucleic acid (DNA) prepared from calf thymus, was obtained from Seravac Laboratories, Cape Town.

Preparation of DNA standard. The standard was prepared by dissolving 5mg. of DNA in 50 ml. 0.005 N-NaOH (100µg.DNA/ml.), and 10 ml. of this solution was diluted to 100 ml. with 50 ml. IN-$HCLO_4$ and 40 ml. of distilled water (10µg. DNA/ml.). During hydrolysis at 70° for 15 min., the solution cleared and was then stored at 4° until used. Standards containing 0.5, 1, 2, 4, 6, 8 and 10µg. DNA/ml. were prepared individually from the hydrolysed solution by diluting it with 0.5N-$HCLO_4$.

Preparation of reagent. To 10 ml. of glacial acetic acid were added 0.15g diphenylamine, 0.15 ml. concentrated sulphuric acid and 0.05 ml. aqueous acetaldehyde (16mg/ml.). The reagent was freshly prepared for each determination, because the blank reading increased if the reagent was stored in the dark at room temperature, as suggested by Burton (1956).

Method. Burton's method was modified for use on the micro scale by using 0.2 ml. of test solution and 0.4 ml. of reagent. Standards containing 0.2-2.0µg. DNA/ml. were prepared as previously described. For the test 0.2 ml. of each standard of 0.5N-$HClO_4$ (blank) and of test solution which contained 0.5N-$HCLO_4$ and had been heated at 70° for 15 min., were mixed with 0.4 ml. reagent and immersed in a water bath at 31.5° for 17 hr. The tubes were closed with clean rubber stoppers. The standards and blanks were prepared in duplicate, and after the heating period the

absorptions of these solutions were measured at 600 mμ against water in the glass micro cells (see spectrophotometry).

Initially the DNA standard stock solution was prepared by dissolving 5 mg. of DNA in 50 ml. 0.005N-NaOH. To 10 ml. of this solution was added 50 ml. IN-$HCLO_4$ and 40 ml. 0.005N-NaOH and the solution hydrolysed as previously described. The addition of 0.005N-NaOH to the diluted solution appeared to be responsible for the calibration curve not passing through the origin, after the subtraction of the blank reading. This problem arose because the blanks contained no sodium hydroxide, and unfortunately this was discovered only subsequent to determining the deoxyribose content of the antigen.

Experimental. Experiments similar to those carried out with the orcinol reaction were repeated with this method. Blanks saturated with chloroform were prepared in duplicate and DNA standards of concentrations varying between 2 and 10 μg./ml. were set up simultaneously. After developing the colour for 17 hr. at 31.5° the standards, blanks and chloroform-treated blanks were measured in the spectrophotometer at 600 mμ. Chloroform had no effect on the reaction at concentrations necessary for preservation of the stock solutions.

Dialysed column effluents, obtained from an agarose pearl column, were treated with the diphenylamine reagent, and after colour development the solutions were measured in the spectrophotometer at 600mμ. Dialysed column effluents did not interfere with the reaction.

Finally, the effect of protein in the form of human serum albumen was investigated. Two solutions were prepared: (a) RNA (6mg.) was dissolved in 25 ml. of water and this solution was diluted 1/10 with water to give a concentration of 24μg. RNA/ml., (b) Human serum albumen (HSA 6mg.) was dissolved in 100 ml. of water, (60μg. HSA/ml.). One millilitre of the RNA solution, and 1 ml. of a mixture of the RNA and HSA solutions in equal volumes, were separately mixed with 1 ml. amounts of IN-$HClO_4$ and the solutions heated at 70° for 15 min. The RNA solution and the RNA+HSA solution were then set up with blanks and standards, and each was mixed with two volumes of reagent. The colour was developed and the absorption measured at 600mμ. Neither RNA nor the RNA+HSA solution appeared to interfere with the reaction.

Calibration lines obtained with tests at concentrations down to 1μg. DNA/ml. were straight and frequently but not invariably passed through the origin (see discussion below), and as the test continued to give reproducible

results it was considered appropriate to apply it to the antigen. For the test on the antigen, 0.2 ml. of each standard (1, 2, 4, 6, and 8 µg.DNA/ml.) and blank 0.5N-$HClO_4$ were prepared in duplicate. To 0.1 ml. of the antigen was added an equal volume of IN-$HClO_4$, and the mixture was heated for 15 min. at 70°. After cooling, the reagent was added to the antigen solution and all the remaining tubes and the colour developed. The values obtained for the standards in this test were so scattered that the reading for the antigen, which was 4.0 µg.DNA/ml., had to be regarded as an indication of the presence of, rather than the concentration of, DNA.

An experiment was done in an attempt to determine whether the value obtained for the antigen was significant. Six blanks and six standards containing 2 µg.DNA/nl. were prepared and the colour developed as before. The standard concentration chosen above was used, for it gave the same absorption reading as the antigen. The extinction of the blanks varied between 0.064 and 0.066, and that of the standards between 0.076 and 0.078, the difference between these two values being the same as the nett reading for the antigen. It therefore seemed probable that the value obtained for the antigen was significant.

In a second experiment, standards, blanks and a further 0.1 ml. of antigen, which were hydrolysed as before, were treated with reagent. The antigen once again appeared to contain 4 µg.DNA/ml. However on this occasion there was good agreement between duplicate standards, and the extinctions of these solutions lay on a straight line, which did not, however, pass through the origin. The results are shown in Table 12 and graphically in Fig. 15.

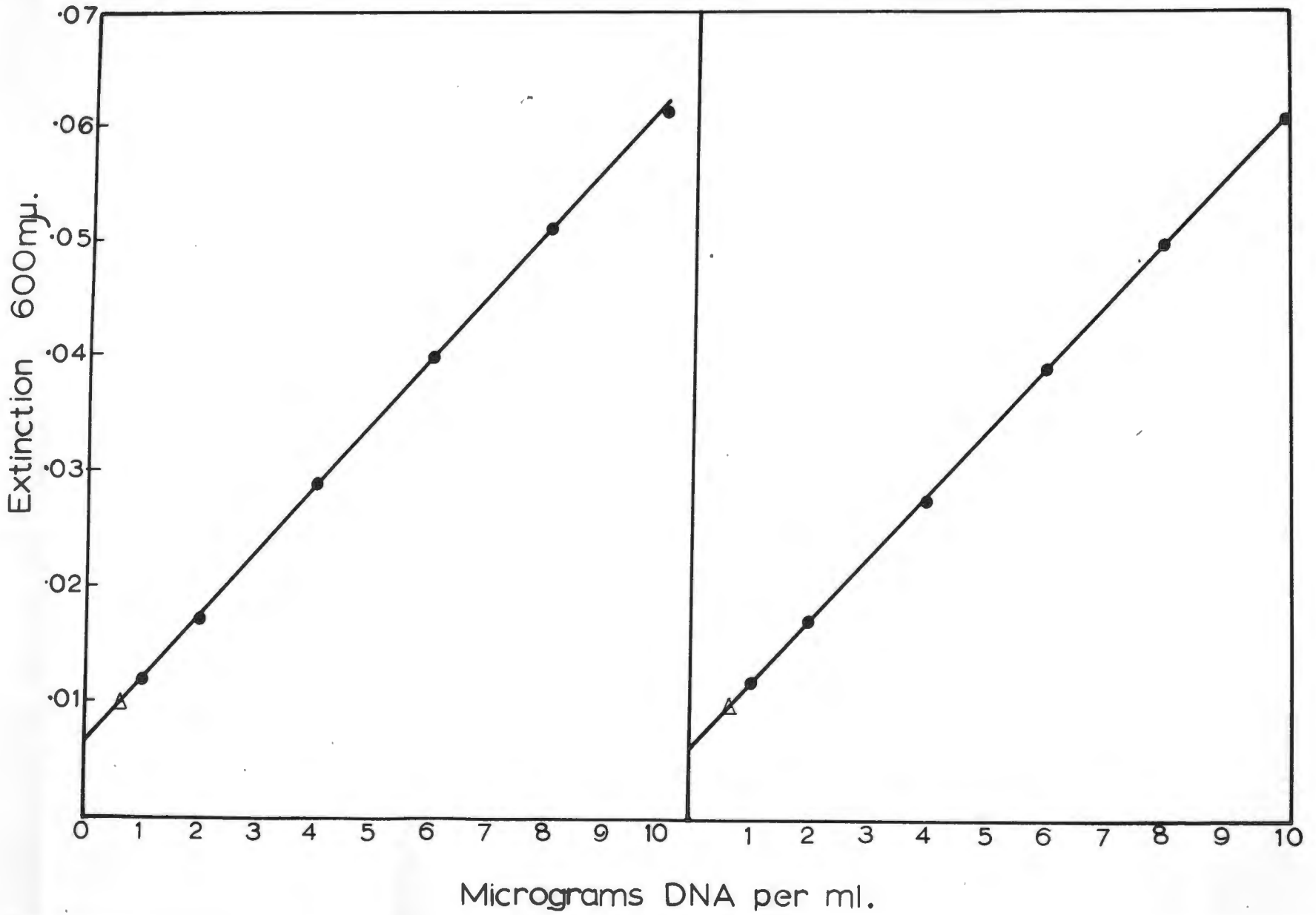
Table No. 12.

Absorption measurements of blanks, standards and antigen solution treated with the diphenylamine reagent.

DNA conc. in µg./ml.	Absorption at 600mµ.	Absorption at 600mµ nett.	Absorption at 600 mµ.	Absorption at 600mµ nett.
Blank	0.056	0	0.057	0
1.0	0.068	0.012	0.069	0.012
2.0	0.073	0.017	0.074	0.017
4.0	0.085	0.029	0.084	0.027
6.0	0.096	0.040	0.096	0.039
8.0	0.107	0.051	0.107	0.050
10.0	0.117	0.061	0.118	0.061
Antigen 0.1ml. + 0.1ml. IN-$HClO_4$.	0.066	0.010		

Fig. 15.

The estimation of deoxyribose in the solution of purified soluble antigen. Extinction at 600mμ (corrected for blank) of duplicate mixtures prepared with standard DNA (hydrolysed) solutions (●——●). Corrected extinction at 600mμ of a single reaction mixture prepared with 0.1ml. of antigen solution and 0.1ml. 1N-$HClO_4$ and hydrolysed as described in the text (Δ).


·07
·06
·05
·04
·03
·02
·01
Extinction 600mμ.
0 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
Micrograms DNA per ml.

Discussion. The diphenylamine reaction of Burton (1956) has been modified to a micro scale for the determination of deoxyribose. But as the sensitivity of this method was not as pronounced as that of the orcinol reaction for ribose, difficulty was experienced in obtaining good agreement between duplicates; as, furthermore, the calibration line sometimes failed to pass through the origin, it was concluded that Burton's method was less well suited than the orcinol test for modification to the ultramicro scale.

Two determinations were done with the antigen, and on both occasions a value of 4 µg.DNA/ml. was obtained. This was considered to indicate the presence of, rather than the concentration of DNA because of the difficulties encountered in the adaptation of the method which have been discussed. Because of the high specificity of the reaction and because false positive results had not been obtained with protein and column effluents, it was considered that some deoxyribose was present in the purified solution of the soluble antigen.

Although these difficulties can be overcome, it is noteworthy that the calibration lines in Burton's paper (Fig. No 1), at the concentration at which these tests were done, curved or did not pass through the origin.

CHAPTER EIGHT.

NITROGEN ESTIMATIONS.

Introduction. Numerous methods exist for the determination of proteins in biological materials. Methods based on the analysis of certain constituents in the protein molecule include the determination of carbon or nitrogen, amino acid content, the colourimetric determination of biuret groups and the formaldehyde binding groups determined by titration. The biuret method estimates the peptide linkages which are approximately proportional to the total protein present, but the method is rather insensitive. Methods for the determination of free amino groups with ninhydrin, and the estimation of phenolic hydroxyl groups by titration are unreliable, owing to the variability in the composition of proteins. Certain proteins contain substances in the molecule, e.g. iron in haemoglobin and iodine in thyroglobulin which are used as a means of determining protein concentration. All the above-mentioned methods are dependant on the purity of the sample, and the constituent under investigation must be present only in the protein molecule and not in any other component present in the material.

There are no methods which are entirely specific for the estimation of proteins as a class of substances, but those

dependant on measuring physical properties are usually sensitive. The refractometric method is not influenced by the composition of the protein and measures the refractive increment due to protein in solution - provided a control is used containing all the other constituents except the protein. Physical methods dependant on the nature of the protein include the spectrophotometric technique which measures the absorption of the protein solution at 280mμ in the ultraviolet light. The measurement of specific gravity, viscosity, surface tension, rotation of polarized light and light scattering have been used for protein estimation (Kirk 1947).

The gravimetric technique consists of precipitating, washing and drying and finally weighing the product. However, the drying must be done under controlled conditions, the point where all the solvent is eliminated and decomposition of the protein begins being difficult to determine. The very sensitive turbidimetric method is not very specific, for some proteins are not precipitated at all. The turbidity caused by the precipitation of proteins, e.g. with perchloric acid, can be measured by absorptiometry or preferably by measurement of the light scattered.

As the percentage of nitrogen in most proteins is remarkably constant, determination of total nitrogen can be used as an accurate means

for the estimation of protein, provided other nitrogenous compounds are eliminated. Nitrogen determinations carried out on biological tissues or fluids have been done for many years by either the Dumas (1881) or the Kjeldahl (1883) methods.

The method of Dumas requires a dry sample of the organic compound which is mixed with copper oxide and heated. The organic compound is oxidized by the hot copper oxide to CO_2, steam and nitrogen and possibly some oxides of nitrogen. A current of CO_2 passed through the apparatus causes the gases of oxidation to pass over a hot copper gauze; this converts the oxides of nitrogen to nitrogen, and the gases are then passed through concentrated alkali solution which absorbs the water vapour and CO_2, and the nitrogen passes on and is collected. This method requires special apparatus, high temperatures and a certain amount of technical skill and is therefore seldom used, even though it is reputed to be more reliable than the Kjeldahl method (Fleck and Munro 1965).

The Kjeldahl method involves the digestion of the sample with hot concentrated sulphuric acid which converts the organic nitrogen to ammonium sulphate; the ammonia can then be determined by several methods. This method is advantageous because either solutions of proteins

or dry samples may be used for investigation. The conditions for the conversion of nitrogen to ammonia must be rigorously controlled if reproducible results are to be obtained. The method has been successfully adapted to a macro (10-30mg.N.), micro (0.5-2mg.N.) and an ultramicro (1-15µg.N.) scale. The digestion procedure involves oxidizing the carbon of the organic matter without oxidizing the ammonia to gaseous nitrogen. The conditions necessary to bring about this conversion include (a) a proper catalyst, (b) the addition of salts to the digestion mixture to elevate the boiling point to the correct temperature of digestion (d) a suitable period of digestion and (e) the possible addition of oxidizing agents to the digestion mixture (Fleck and Munro, 1965). The above variables must be rigorously controlled to prevent the decomposition of ammonia to gaseous nitrogen.

At least 40 different catalysts have been investigated for the Kjeldahl determination of nitrogen (Fleck and Munro 1965). Mercury appears to be the most effective, but on the addition of alkali to an ammonium solution containing mercury a considerable fraction of the ammonia is bound by the mercuric oxide precipitate. To overcome this problem sodium thiosulphate is added to the digest. McKenzie and Wallace (1954) found the optimum concentration of HgO to be between

40-50mg/ml. of concentrated H_2SO_4.

Potassium phosphate, phosphoric acid, sodium sulphate or potassium sulphate are added to the digestion mixture to elevate the boiling point. Potassium phosphate and phosphoric acid are not satisfactory as they etch the glassware (Hiller, Plazin and van Slyke 1948), but potassium sulphate has been found by Fleck and Munro (1965) to be the most satisfactory.

Numerous investigations have been done to determine the optimum temperature for complete digestion of organic material including refractory organic substances without loss of nitrogen. The digestion of organic material in open tubes has been described by McKenzie and Wallace (1954). Grunbaum, Kirk, Green and Koch (1955), have devised a technique whereby the samples are digested in sealed tubes at 400°-440° in the absence of catalysts. The most effective means of controlling the temperature is by varying the concentration of K_2SO_4 in the digestion mixture (Fleck and Munro 1965). McKenzie and Wallace (1954) have suggested the addition of 0.65g. K_2SO_4/ml. of sulphuric acid to give a temperature of 360°, or the addition of 1g K_2SO_4/ml. sulphuric acid to give a temperature of 390° when boiled. For the micro Kjeldahl

method, Fleck and Munro (1965) have suggested 1.2g K_2SO_4/1.5ml. H_2SO_4 for refractory organic substances. The time required for complete digestion is inversely proportional to the temperature of the digest (McKenzie and Wallace, 1954).

The addition of oxidizing agents has been suggested for samples containing a high percentage of organic carbon. Potassium permanganate was successfully used by Beet (1955) for oxidizing coal. Moore and Diehl (1962) have used perchloric acid, but care should be exercised when using this powerful oxidant, for it is very liable to cause the decomposition of ammonia. Using hydrogen peroxide as an oxidant, McKenzie and Wallace (1954) reported no loss of nitrogen; they have attributed this to the low temperature of decomposition of hydrogen peroxide (160°), and secondly to the cooling of the digest prior to the addition of the oxidant.

The ordinary Kjeldahl digestion procedure is not adequate for the estimation of nitrogen in nitrates, azo-compounds and nitrose compounds. These compounds must undergo a preliminary reduction, either by hydrogenation or by the addition of a reducing agent prior to the digestion process (Fleck and Munro 1965). Azo and nitroso compounds, nitrites etc., should be reduced with formic

acid in the presence of zinc and iron according to the method of Dickinson as cited by Fleck and Munro (1965).

The ammonia may be separated from the digest after treatment with excess alkali by aeration, diffusion or distillation. The original steam distillation process involved collecting the distillate in standard acid, and determining the ammonia by back titration with NaOH. Alternatively the excess acid can be determined by iodimetry or the ammonia may be collected in boric acid and titrated directly with HCl. Alternately one of the colourimetric methods described below can be applied to the distillate.

Aeration and diffusion methods are convenient when many estimations must be done at the same time. The excellent review by Fleck and Munro (1965) deals with the special apparatus and techniques necessary for this method.

Colourimetric methods are usually applied directly to the digest in the preliminary separation of the ammonia, and include (a) the Nessler reaction (b) the reaction of ammonia with phenol and hypochlorite and (c) the reaction with ninhydrin.

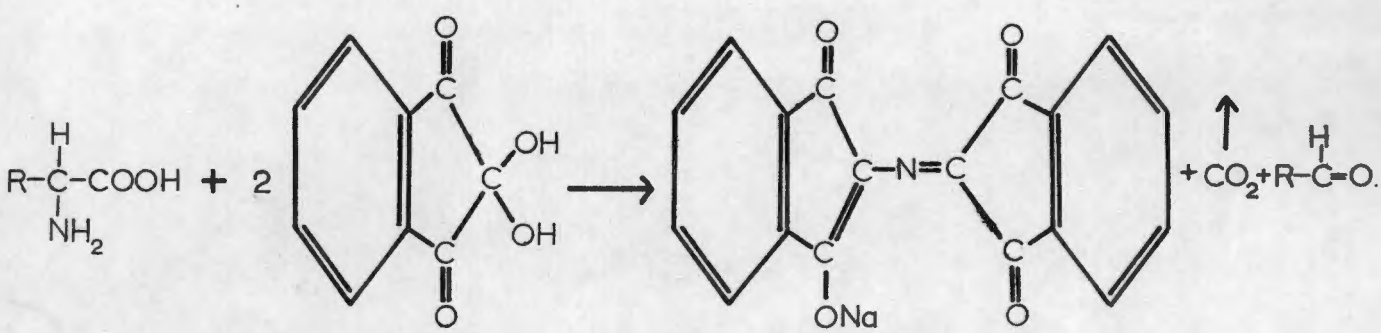
The Nessler reagent (potassium iodide and mercuric iodide) is added to the distillate or directly to the digest prior to the addition of sodium hydroxide.

The yellow-brown colour produced during this reaction is due to a mercury-iodine-ammonia complex (Fleck and Munro 1965). The reaction is sensitive to a number of variables, (Thompson and Morrison 1951), which include the preparation and maintenance of the reagent, the conditions during digestion, the temperature of the sample during Nesslerization, and the alkalinity of the sample before and after nesslerization. Thompson and Morrison's experiments have shown that the Nessler reagent deteriorates on storage, but that it could be restored to its original state by the addition of a solution containing potassium iodide and iodine.

A method based on the reaction between ammonia, phenol and hypochlorite was reported by Russell (1944). A modification of this method (Mann 1963) for the determination of nitrogen (1-15μg) depended on the digestion of the sample with sulphuric acid in the presence of potassium sulphate and mercuric oxide, and the subsequent treatment of this digest with zinc, followed by neutralization. The phenol-hypochlorite reagent was then added directly to the digest and the ammonia determined colourimetrically.

The colour reaction between ninhydrin and ammonia, amino acids, peptides, proteins and other

substances containing free amino groups has been studied extensively. Moore and Stein (1948) developed a technique for the reaction of ninhydrin with amino groups to give diketohydrindylidene-diketohydrindamine as the basis for the photometric determination of amino acids in effluents from starch chromatograms. The reaction is believed to be as follows:



α-amino acid ninhydrin diketohydrindylidene-diketohydrindamine

Furthermore, they found that the colour produced during the test was reproducible if hydridantin or stannous chloride was added to the reagent to eliminate oxidative side reactions. The reaction was done at pH 5.0 and 100°, and the absorption maximum of the blue product formed was at 570mμ. In a subsequent paper Moore and Stein (1954) modified their reagent by the addition of hydridantin because it was found that stannous chloride precipitated

in the reaction mixture.

Matheson, Tigane and Hanes (1961) developed an improved ninhydrin-hydrindantin reagent for the qualitative determination of amino acids and peptides separated on filter paper chromatograms. The reagent is claimed to have a high stability, low blank readings and stoichiometric yields of Ruhemann's purple for most amino acids. The reagent could also be applied to solutions containing amino acids.

Jacobs (1959) digested dried samples of proteins with sulphuric acid in the presence of copper sulphate, potassium sulphate, mercuric oxide and selenium, and mixed the neutralized digest with a 2% solution of ninhydrin containing stannous chloride prior to heating at a 100° for 30 min. Micro and ultra micro modifications of this method were described later, (Jacobs 1960). Digestion of dried or solid samples with sulphuric acid in sealed tubes was done between temperatures of 460°-480°, but at 560° a loss of nitrogen was noted. Furthermore, if the 4% stock solution of ninhydrin in methyl cellosolve was treated with a cation exchange resin, the colour yields were always consistant. The method has been found satisfactory for the determination of nitrogen in serum, protein hydroly-

sates and heterocyclic compounds.

Jacobs (1962) modified the method even further by eliminating the use of catalysts. Nitro compounds, after reduction, were analysed successfully by this method, the limit of sensitivity being dependent on the accuracy of sampling. Finally, Jacobs (1964) reported that quantitative recoveries of nitrogen were temperature dependent, the optimum being between 460°-480°. The presence of a catalyst was found to be unnecessary, if the period of digestion was adequate.

Of the methods included in the above brief review that of Jacobs was adopted for determining the nitrogen content in the solution of the rabies soluble antigen. The method is advantageous in that it is sensitive, applicable to micro-volumes of test solution, that no salts need be added to elevate the boiling point and that the use of catalysts is eliminated.

MATERIALS AND METHODS.

Nitrogen determinations on the purified antigen solutions were performed according to the method of Jacobs (1956, 1959, 1960, 1962 and 1964), with minor modifications which are described in this section.

Materials.

Ninhydrin (Pierce Chemical Co., Rockford, Illinois, U.S.A.) was used without further purification.

Methyl Cellosolve (2-methoxy ethanol BDH). One litre of methyl cellosolve was redistilled under vacuum after the addition of 10 ml. of a solution prepared by dissolving 50g $FeSO_4.7H_2O$ in 100 ml. 2.0M-H_2SO_4, to remove peroxides (Jacobs 1960). However, this treatment was not successful in lowering the blank reading, which was high probably due to some impurity in the methyl cellosolve other than protein. A sample of peroxide free methyl cellosolve (Union Carbide Chemical Co., New York, U.S.A.) was found to give a lower blank reading without further treatment, and it was consequently used throughout these investigations. Samples of methyl cellosolve were tested periodically for the presence of peroxides according to the method of Moore and Stein (1954). To 2ml. of methyl cellosolve was added 1 ml. of freshly prepared 4% ($^{w}/_{v}$) aqueous potassium iodide, which always gave a colourless solution indicating the absence of peroxides.

Sulphuric acid. (Microanalytical reagent BDH) was used without further purification.

60% ($^{w}/_{w}$) Sodium hydroxide. Sodium hydroxide (60g.) was dissolved in distilled water (40 ml.). In a preliminary titration, 20 µl of concentrated sulphuric acid was diluted with citrate buffer pH 5.0 (2.0M, 0.5ml.) and water to 5ml.; this required 20 µl of NaOH (60% $^{w}/_{w}$) to bring the pH to 5.5.

Stannous chloride dihydrate (Analar BDH) was recrystallized from warm 2N-HCl. A solution of the salt in warm 2N-HCl was centrifuged at 2,500 rev./min. for 10 min. to pellet the insoluble impurities, and the pure salt was allowed to crystallize from the clear supernatant fluid. The crystals were washed with cold 2N-HCl and air dried.

Ethyl alcohol (Analar BDH) was used without further purification.

Citric acid (Analar BDH) was recrystallized from water, as this treatment assisted in reducing the blank reading.

Acetic acid. (Pro-analysi Merck) was redistilled before use.

Sodium acetate trihydrate (Analar BDH) was recrystallized from water.

4M Sodium acetate buffer pH 5.5 was prepared by dissolving sodium acetate trihydrate (108.8g in warm water (100 ml.). Acetic acid (20 ml.) was added to the cooled solution,

which was diluted to 200 ml. with water (Jacobs 1960).

2.0M Sodium citrate buffer pH 5.0 was prepared by dissolving NaOH (80g) and citric acid (180g) in 1 litre of water (Jacobs 1960).

Dowex 50-X cation exchange resin (Fluka-Chemische fabrik Buschs) was converted to the hydrogen form by treatment with 4N-HCl, after which it was transferred to a No 2 sintered glass filter and washed with distilled water until the pH of the filtrate was about 5.5. The resin was then washed with methyl cellosolve before it was added to the stock ninhydrin solution (Jacobs 1956).

Preparation of stock ninhydrin solution. Ninhydrin (4g.) was dissolved in peroxide free methyl cellosolve (150 ml.) and the dowex resin (prepared as above) was added. The flask containing the ninhydrin solution was evacuated with periodic shaking to liberate the oxygen bubbles trapped in the resin, and refilled with nitrogen. This solution was stored at 4^{c} until used, and it could be kept for at least a month without apparent change.

Preparation of the ninhydrin reagent. A reagent containing 75% methyl cellosolve instead of the 50% prescribed by Jacobs (1960) was used, because it remained clear after the addition of the stannous chloride. A mixture of the stock ninhydrin solution (30 ml.) and 4.0M acetate buffer

(pH 5.5, 10 ml.) was freed of dissolved oxygen by subjecting it alternately to a vacuum and an atmosphere of nitrogen. This procedure was repeated twice. Stannous chloride (16 mg.) was added and oxygen removed as before. The slightly red solution was kept in the dark for about 2 hr., by which time the reddish tinge had almost disappeared. Solutions used immediately after the addition of stannous chloride gave erratic results. The reagent was filtered after the two hour period, through a No 4 sintered glass filter and used immediately (Jacobs 1960).

Preparation of hydrindantin. The method of Moore and Stein (1954) was followed. To a solution of ninhydrin (20g.) in water (500 ml.) at 90° was added with stirring an aqueous solution of ascorbic acid (20g.) in 100 ml. at 40°. When the solution had cooled to room temperature the hydrindantin was collected and washed with cold distilled water. The product was dried to constant weight over phosphorus pentoxide in an evacuated desiccator which was kept in the dark as the substance was light sensitive.

Ammonium sulphate standards. A solution was prepared containing 100µg.N/ml. by dissolving ammonium sulphate (47.21mg.) in distilled water (100 ml.). Further dilutions were prepared in water when required.

Preparation of glycine standard. Glycine was recrystallized

twice from water. A standard stock solution was prepared by dissolving glycine (53.62 .) in distilled water (100 ml.). This solution (100 .N/ml.) was diluted further with water when required.

Calibration of the Chromel-alumel thermocouple.

The thermocouple junctions were kept in glass tubes. The zero position of the galvanometer (Cambridge Instrument Co., England) was set with the "hot" and "cold" junctions of the thermocouple immersed in a mixture of ice and water, with the galvanometer shunt set to give 1/10 of the full sensitivity. The cold junction was left in position and the hot transferred to a test tube approximately 1 cm. above the surface of boiling sulphur, i.e. directly in the vapour. Once the galvanometer reading was constant the reading was noted. The procedure described above was repeated with boiling mercury, diphenyl, aniline and water. The galvanometer readings and temperatures are recorded in Table 13 and graphically in Fig 16.

Fig. 16.

Calibration of the chromel-alumel thermocouple using substances of different boiling points (●——●). Plot of the galvanometer readings against the temperature in degrees centigrade. Temperature of muffle furnace for digestion of samples (▲).

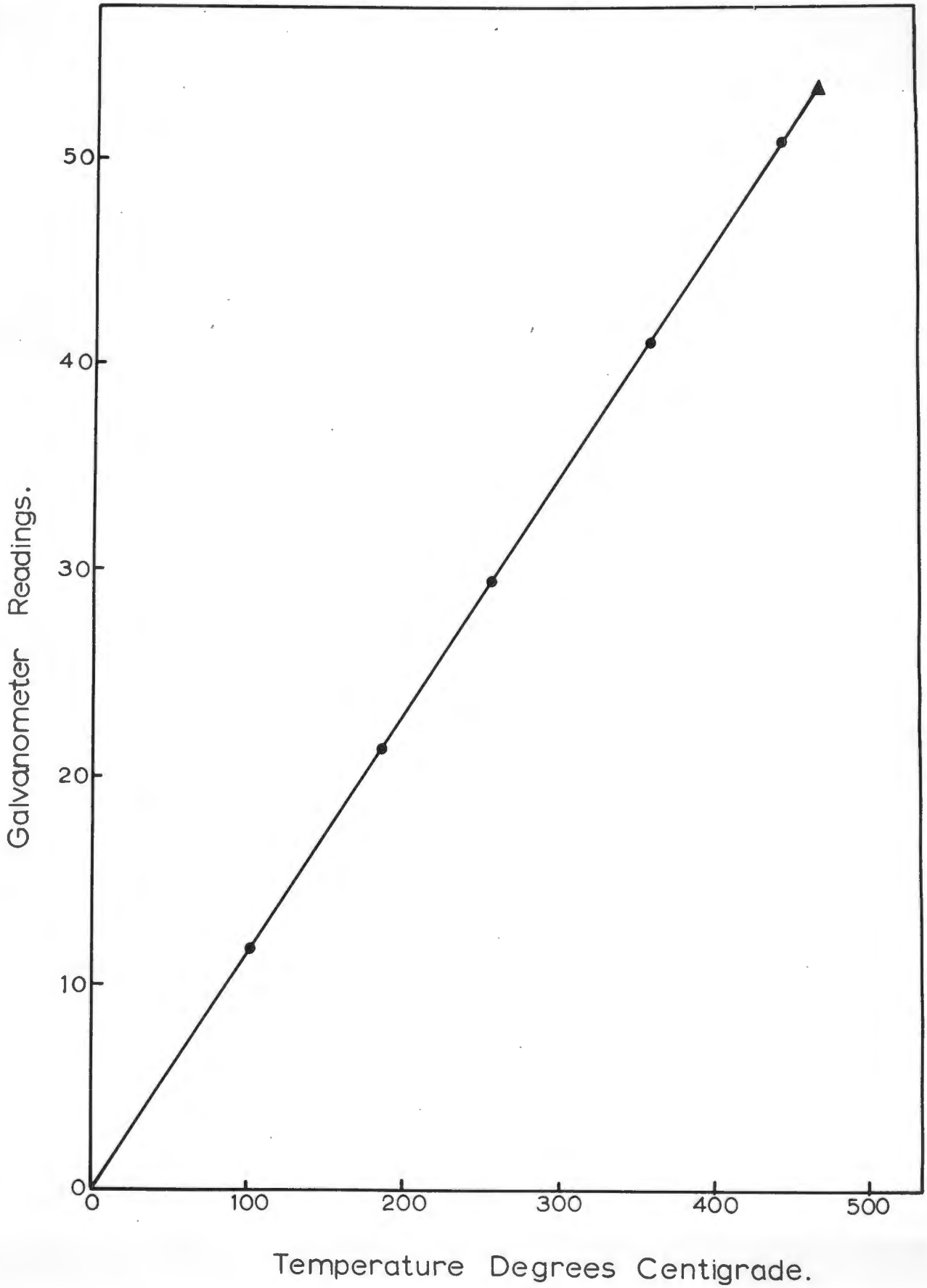

Galvanometer Readings.
0
10
20
30
40
50
0
100
200
300
400
500
Temperature Degrees Centigrade.

Table No. 13.

Galvanometer readings obtained with a chromel-alumel thermocouple using substances of varying boiling point.

Substance	Temperature 0^{o}	Galvanometer reading
Ice	0	0
Sulphur vapour	445	51
Mercury vapour	357	41.3
Diphenyl vapour	254	29.1
Aniline vapour	184	21.2
Steam	100	11.6

After calibration, the tube containing the hot junction was fixed in the furnace so that the junction was in the centre of the muffle (Gallenkamp & Co., London).

Method. In preliminary experiments to determine the relationship between the amount of nitrogen in the sample and the colour produced, tests were set up in duplicate. Tubes (5 x 70 mm.) sealed at one end received 0.10 ml. of water (blank) and standard solutions (8, 16 and 32 µg.N/ml.). The tubes were transferred to an oven at 90° in order to evaporate their contents to dryness. To each tube containing the dried sample were added 20 µl of sulphuric acid using an Agla micrometer syringe (Burroughs Wellcome & Co., England). The tip of the glass needle of the syringe was bent at a right angle, to facilitate the delivery of the

acid down the side of the tube. The tubes were sealed and when the furnace had been maintained at 470° for at least 1 hr. the tubes were placed in it as close to the hot junction of the thermocouple as possible. After 30 min. the furnace was switched off and the tubes allowed to cool overnight.

The tubes were opened and both halves of each tube well washed with distilled water, the washings being transferred to a 5ml. volumetric flask. Citrate buffer (pH 5.0, 2.0M, 0.5 ml.) and 20µl NaOH (60% $^{w}/_{w}$) were added to each flask and the contents made up to 5 ml. with water and shaken vigorously. Two millilitres of the contents of each flask were pipetted into a series of 10 ml. flasks, to each of which was added 2 ml. of ninhydrin reagent. The flasks were capped with glass shells and immersed in a boiling-water bath for 30 min. The flasks were cooled in tap water and the contents were diluted to the mark with 50% ($^{v}/_{v}$) ethanol. The absorption of each solution was measured against water in a 2 cm. glass cell at 570mµ. On the following day a second sample (2 ml.) was removed from each 5 ml. flask, and the colourimetry repeated using a freshly prepared ninhydrin reagent.

Experimental. Initially a certain amount of difficulty was encountered for duplicate samples were not in agreement with

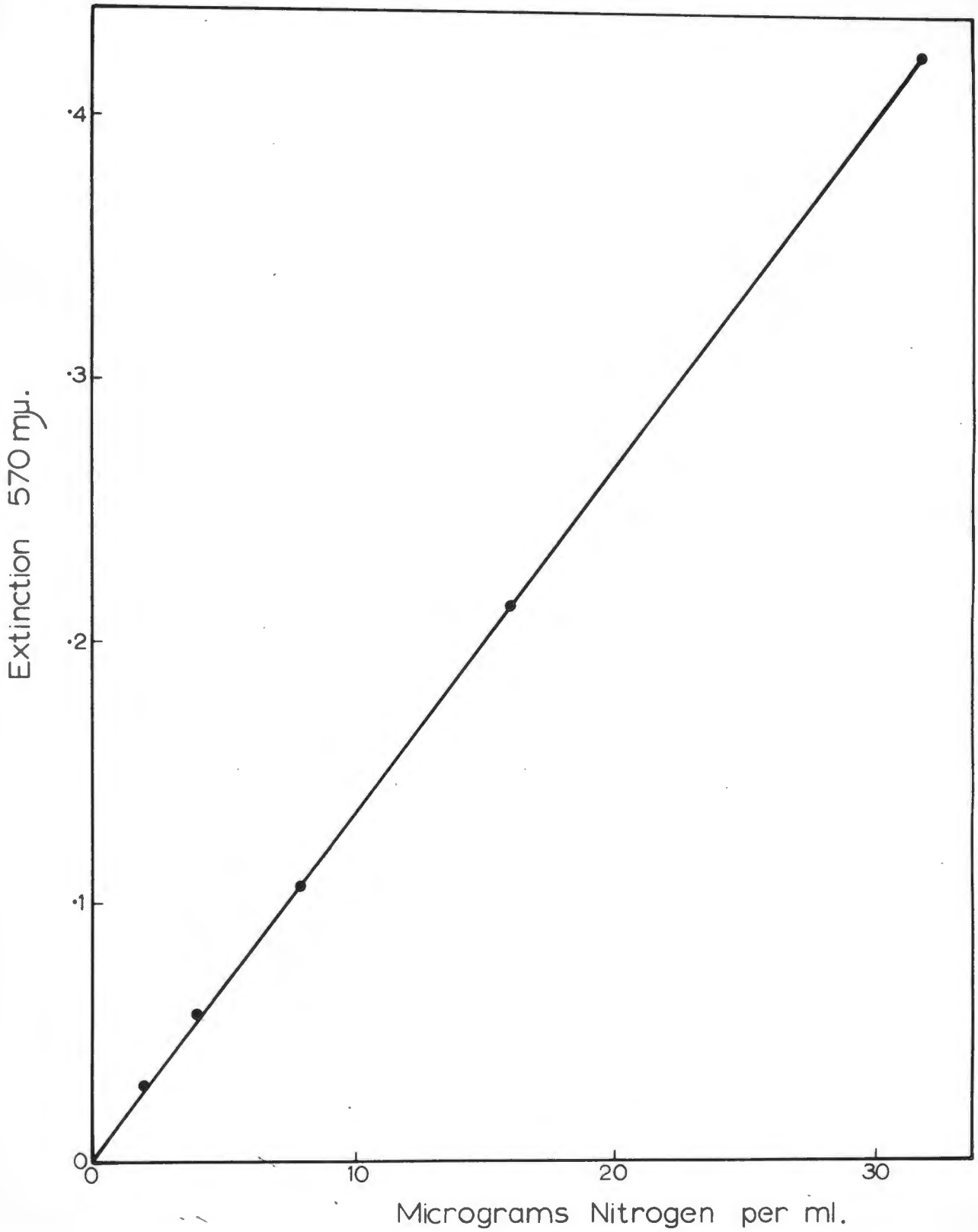
each other. It was noticed on numerous occasions that the solutions became cloudy after the 30 min. heating period. In all probability the cloudy appearance of the solutions was due to the stannous chloride, and in an attempt to overcome this problem the stannous chloride was recrystallized, but this step did not eliminate the turbidity. Stannous chloride obtained from different manufacturers was investigated, but there was no improvement. Ultimately it was found that by increasing the concentration of methyl cellosolve to 75% in the reagent the turbidity was completely eliminated.

One of the disadvantages of this method is the relatively high readings obtained for the blanks. This reading was reduced by recrystallizing the citric acid from water and thereafter reducing the concentration of the stannous chloride recommended by Jacobs, by approximately a third. Redistillation of the acetic acid and recrystallization of the sodium acetate used in preparing the acetate buffer gave no improvement in the blank reading.

A solution containing ninhydrin and hydrindantin, (but no stannous chloride) was found to be more sensitive than that prepared with stannous chloride, but the blank readings were as high or higher than those obtained with the original reagent.

Fig. 17.

Estimation of total nitrogen using blanks and ammonium sulphate standards of known nitrogen content (●——●).


Extinction 570 mμ.
·4
·3
·2
·1
0
0
10
20
30
Micrograms Nitrogen per ml.

Standards (0.10 ml.) of ammonium sulphate were prepared containing 2,4,8,16 and 32µg.N/ml. The digestion procedure was omitted and the citrate buffer alone was added to each sample. The colourimetry was then carried out on each sample. The results are recorded in Table 14 and graphically in Fig. 17.

Table No. 14.

Nitrogen determination using ammonium sulphate standards.

Nitrogen conc. in µg./ml.	Absorption at 570mµ average of 2 readings.	Corrected reading at 570mµ.
Blank	0.09	0
2	0.122	0.032
4	0.149	0.059
8	0.197	0.107
16	0.305	0.215
32	0.515	0.425

An experiment similar to that described above was done using glycine standards which were digested with sulphuric acid. The samples for colourimetry were prepared as described in methods. The results are recorded in Table 15 and graphically in Fig. 18.

Fig. 18.

Estimation of total nitrogen using blanks and glycine standards of known nitrogen content (●——●).

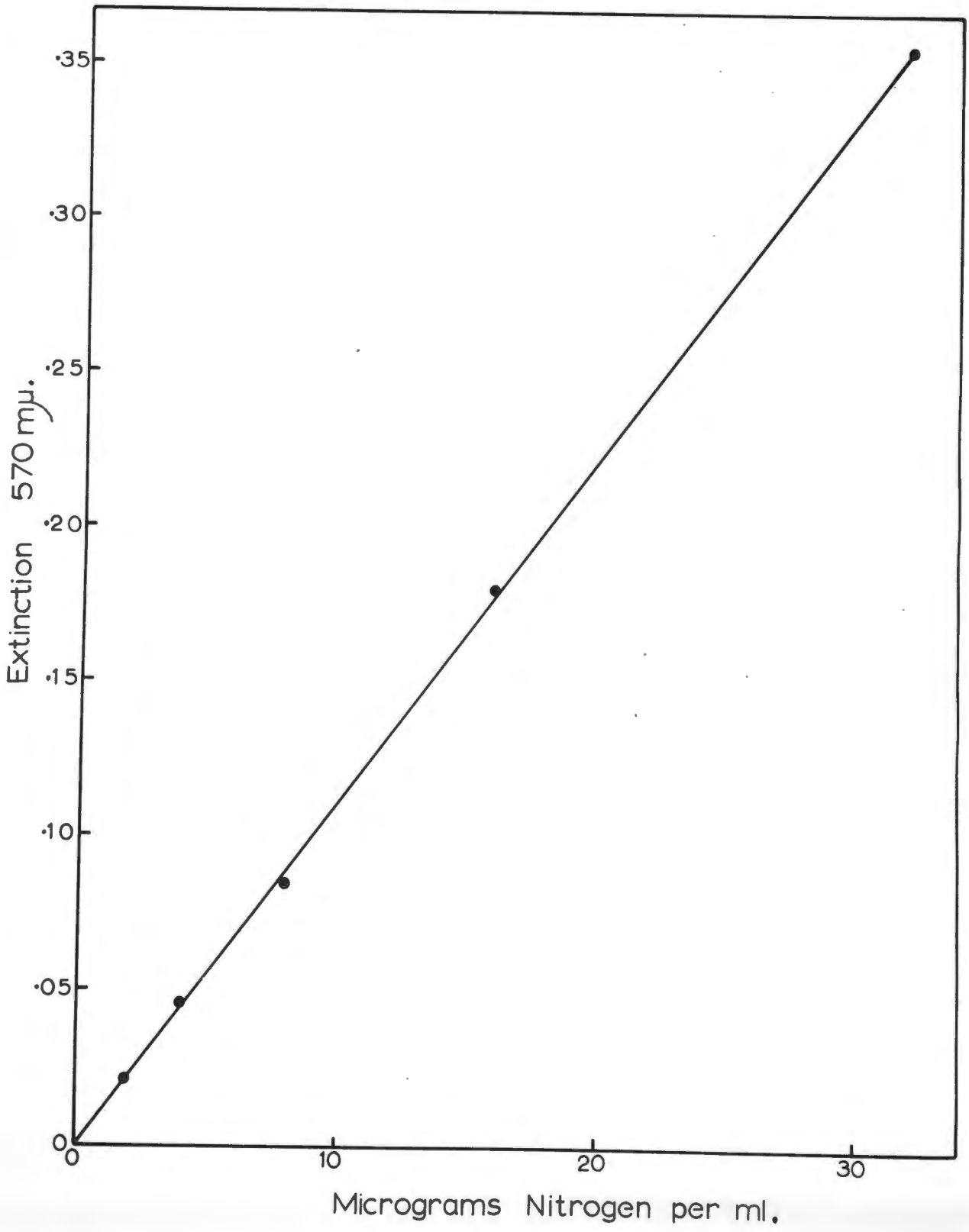

·35
·30
·25
·20
·15
·10
·05
0
Extinction 570 mμ.
0
10
20
30
Micrograms Nitrogen per ml.

Table No. 15.

Nitrogen determination using pre-digested standards of glycine.

Nitrogen conc. in µg./ml.	Absorption at 570mµ average of 2 readings.	Corrected reading at 570mµ.
Blank	0.087	0
2	0.106	0.019
4	0.134	0.047
8	0.170	0.083
16	0.268	0.181
32	0.441	0.354

From the results of the previous experiments it was found that the high blank readings were not due to the sulphuric acid or the sodium hydroxide, for the blank values for both experiments were in good agreement with each other. Secondly, the digestion procedure was found to be adequate, using glycine standards. The efficacy of the digestion procedure was further tested using standards of histidine, and the results were in fair agreement with the theoretical values. As the test continued to give reproducible results the antigen solution was finally investigated.

Duplicate blanks and glycine standards containing 8, 16 and 32µg.N/ml., were prepared. The antigen solution (0.10 ml.) was pipetted into one tube and all the solutions

dried and treated as previously described. The colourimetry was repeated the following day using the samples prepared the previous day, except that the reagent was freshly prepared. The results for both determinations are recorded in Table 16 and graphically in Fig. 19.

Table No. 16.

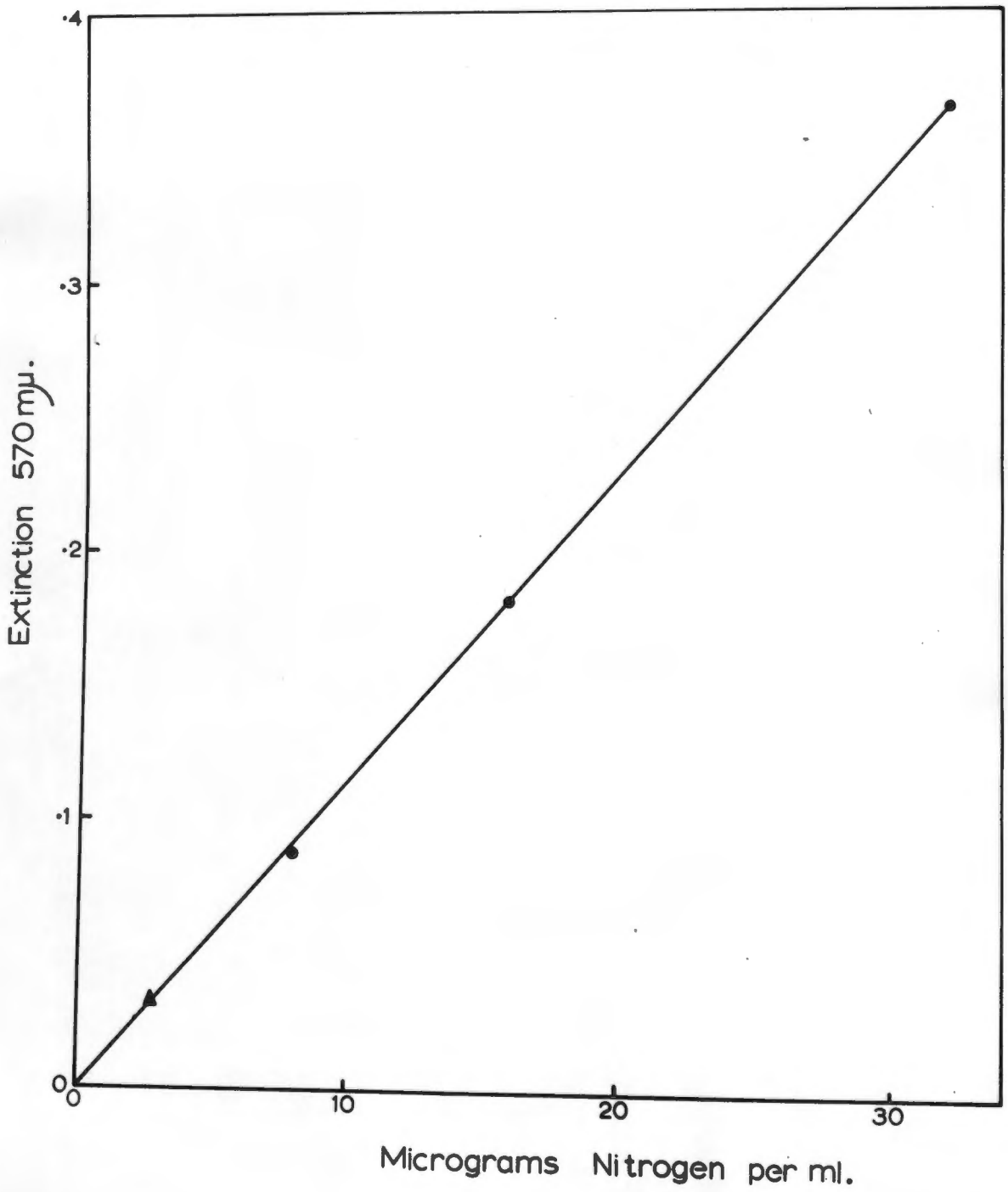
Nitrogen determination of glycine standards and purified soluble antigen solution.

Standard and antigen conc.	Absorption at 570mμ Mean of 4 readings.
Blank	0.131
8μg.N/ml.	0.083
16μg.N/ml.	0.181
32μg.N/ml.	0.369
0.1 ml. antigen	0.033

Discussion. The determination of nitrogen in the purified solution of the soluble antigen was done according to the method of Jacobs (1956, 1959, 1960, 1962 and 1964). It was found necessary to introduce certain modifications because the method did not give reproducible results; these have already been described in the text. The antigen solution appeared to contain about 3μg.N/ml. (see Fig 19). From

Fig. 19.

Estimation of total nitrogen in the solution of purified soluble antigen. The digests were set up in duplicate and the ninhydrin colourimetry on each digest was repeated with fresh reagent on the following day to give four values for the extinction of the blank, the antigen (▲) and each standard (●——●).


·4
·3
·2
·1
0
Extinction 570 mμ.
0
10
20
30
Micrograms Nitrogen per ml.

previous experiments it was deduced that the antigen contained 7.3µg. RNA/ml. of which 16.3% was nitrogen, and this would consequently account for 1.18µg.N/ml. After subtraction of this value from the total nitrogen concentration, (3µg./ml.), 1.82µg.N/ml. would remain. If this nitrogen is derived from a protein containing 16% nitrogen, 11.4µg./ml. of protein would be present, but as some DNA may also occur in the antigen this figure must be taken as the upper limit.

CHAPTER NINE

CONCLUSIONS.

Extracts of normal and rabies-infected suckling mouse brain suspensions were purified (Mead 1962a, b, Katz, Larsson and Mead 1967) by acid precipitation, digestion with nucleases and trypsin, preparative centrifugation, exclusion chromatography and iso-density gradient centrifugation. After this treatment the large antigen was considered to be relatively pure, for all the constituents seemed to have been eliminated from extracts of normal brain material during the purification process. The large antigen was rabies specific, as was shown by complement fixation and gel precipitin tests using mouse antisera which had failed to reveal any specific reaction with normal brain extracts by these methods.

The term soluble antigen has been applied to all antigenic components other than complete virus particles formed in or by virus infected but not by normal cells. These particles, which vary in size according to the virus system, are capable of being broken down into smaller units; under certain conditions the smaller antigens can aggregate to form larger complexes (Kipps, 1958).

Antigens produced by viruses from different groups appear to be unrelated, but those produced by viruses within the same group appear in some cases to be group-specific antigens (Kipps, 1958), while others are type-specific. It seems therefore that there is no connection between antigens produced by different virions.

Soluble antigens may be constituents of virions which are (a) not yet assembled into complete units, (b) mistakes or rejects and (c) produced by disintegration of the preformed virions. Secondly, soluble antigens may not be constituents of the virus particle and may therefore be (a) special enzymes (b) structures used as building bricks for the production of new virus particles and (c) products of infection not having any special significance.

Craigie (1932) was the first to recognize the existence of a non-infectious particle (soluble antigen) which he found present in suspensions of vaccinia virus. Shortly after, Smith (1936) found that suspensions of influenza virus contained antigens separable from the virus which fixed complement in the presence of immune sera. These findings stimulated considerable interest in the particles.

It has been suggested by Wiener, Henle and Henle (1946), that the soluble antigens of influenza virus may be formed during the disintegration of virions by intense sonic vibrations. Though the antigens may have originated from disrupted virions they are also found in rabies virus suspensions which have not been ultrasonicated (Van den Ende, Polson and Turner 1957).

The soluble antigen of influenza virus was found to be a nucleo-protein by Hoyle (1952), a claim which was supported by Ada and Perry (1954) who found that the antigen contained RNA. The last-named workers suggested that this antigen was a "non-infective immature form or building block" which was later converted into fully active virus particles. However, this was not true for the soluble antigens of rabies virus, for Van den Ende, Polson and Turner (1957) found that the titre of the soluble antigen increased with increasing virus titre, which would not have been expected if they were indeed being converted at a later stage to fully active virions.

Wildy and Holden (1954) have suggested from their studies with the soluble antigens of herpes simplex virus that these particles may represent a matrix or template used for the multiplication of infectious virus. This assumption may be correlated with the work of Pace and

Spiegelman (1966) who have shown that the incoming viral RNA acts as a template for the replicase which in turn is responsible for the production of new infectious RNA molecules. Their results did not support the hypothetical activity of a pre-existent RNA molecule or of some other component necessary for the production of new virus particles.

Finally, the theory of Burnet and Lind (1951) postulates that the soluble antigen is a replecating genetic unit or possibly a misfit incapable of being included in infectious virus particles. A possible example could be the short rods of tobacco mosaic virus (TMV) which are non-infectious but have an RNA: protein ratio and serological specificity similar to that of the complete rods of TMV (Commoner, Shearer and Yamada 1962).

During the present investigation, the largest antigen of rabies virus, was found to contain RNA, protein and possibly DNA, the concentration of which could not be determined accurately. The ultraviolet absorption curve (Fig. 5) also indicates that the antigen is a nucleoprotein, containing about 5% of nucleic acid. The ribose and nitrogen determinations however, showed a concentration of RNA between 20% and 40% or even more, depending on the proportion of purine-bound ribose in the nucleotide.

The appearance of the antigen is shown in the electron micrograph; (Fig. 6) it appears to be composed of rings or single turns of a helix.

Pinteric, Fenje and Almeida (1963) have shown that rabies virus contains one or more helical structures, which they suggest are ribonucleoprotein. Similarly, Bradish, Brooksby and Dillon (1956) found that the infective particle of vesicular stomatitis virus was a coiled "beaded filament or platelet stack". They have suggested that the two complement fixing components (20S and 6S) are fragments produced by the disruption of this filament. Their electron micrograph reveals a few rings similar to those found in the rabies antigen photograph. These rings were also seen in the pictures published by Pinteric et al (1963) but in both these cases the rings were not as prominent a feature as they were in the electron micrograph of the rabies soluble antigen. It is tempting to suggest that the rabies antigen rings are possibly single turns of the helix believed to be contained within the virus, as their diameters are very nearly the same as the tightly coiled helix of the virus; but this assumption is in disagreement with certain known facts. Firstly, the virus helix is seen to be unwinding from the disrupted virus particle

rather than breaking down into rings. It could be argued that the antigen rings arose due to the breakdown of the helices during extraction or acid precipitation at pH 4.5 (Katz, Larsson and Mead 1967). From the work of Van den Ende, Polson and Turner (1957) these assumptions are not in agreement with their findings, as they found that virus suspensions freed of soluble antigen when ultrasonicated for 6-36 min. or if frozen and thawed, released a very small fraction of soluble antigen. This small release of antigen would not have been expected if the antigen was a constituent of the virus released by its disruption. It could have arisen from tissue material in the suspension. This suggests, but does not prove, that the antigen is in fact a non-essential constituent of the virus. Furthermore, it is unlikely that the breakdown of the helix took place, if indeed it did, during digestion with nucleases or trypsin, for the antigen was identical before and after digestion as shown by gel precipitin tests (Katz, Larsson and Mead 1967).

The possibility of the antigen rings arising as a stage in the formation of complete helices must now be discussed. Unfortunately there is no evidence to support this for there is no indication in the micrograph to suggest that helices are in fact being produced. It would have seemed feasible that at least one or more short coils would

have appeared in one of the numerous electron micrographs taken during these experiments, but none were observed. It is conceded that although these short coils have not been found this is not sufficient evidence to exclude the possibility that they arose during multiplication, for they may have been removed during the purification process.

The soluble antigen was found to be resistant to digestion with nucleases, trypsin and chymotrypsin. The double stranded RNA of reovirus (Gomatos and Tamm 1963) and the apparent triple stranded RNA of poliovirus (Bishop, Summers and Levintow, 1965) are both resistant to RNAase digestion. If the antigen consists of a double or triple stranded RNA molecule this would possibly explain its resistance to digestion by RNAase. Even though numerous attempts to stain the antigen-antibody precipitates fluorescent red with acridine orange have failed, (Katz, Larsson and Mead 1967), this is not conclusive evidence to support double or triple strandedness.

Bassel, Hayashi and Spiegelman (1964) have prepared from a single stranded DNA molecule, by enzymatic synthesis, a circular DNA-RNA hybrid which is RNAase resistant. Initially it was thought that the soluble antigen of rabies was comparable with this complex, but as their appearance in electron micrographs was so dissimilar and the disparity between the sizes so great,

it seemed unlikely that there was any connection between them.

Influenza virus particles are resistant to digestion by trypsin and RNAase unless they are pretreated with 0.1N-HCl (Valentine and Isaacs, 1957a,b). After the acid treatment the particles are sensitive to trypsin digestion, and to RNAase after trypsinizing. The product of digestion had the form of rings of approximately the same diameter as the original particles. The rabies antigen on treatment with 0.1N-HCl for 30 sec. was completely destroyed, prior to the addition of trypsin and RNAase.

Patel and Wang (1965) isolated an active complex of DNA-RNA-protein capable of incorporating C^{14} tryptophan into its non-histone proteins. The chemical analysis of these particles revealed that they contained 35% DNA, 2% RNA and 63% protein. An electron micrograph showed that they consisted of spherical particles measuring 10 to 30 in diameter, many of which were associated with strands of DNA 3.2-4.0 mμ in diameter.

In a subsequent paper, Wang (1965) showed that these residual proteins, isolated from normal calf thymus tissue, had sedimentation coefficients of 4S, 11S, 13S, 17S and 22S. The slower sedimenting particles were

in the form of spheres (5-25μ in diameter) and most of the RNA was incorporated in them.

It would be plausible to regard the rabies soluble antigen as a RNA-protein-DNA molecule capable of synthesizing infectious RNA of rabies virus, or of synthesizing virus protein.

Evidence that host cell DNA may be implicated in the synthesis of some RNA viruses depends mainly on the effect of actinomycin D on virus production. Evidence of this effect has lost some of its force following the discovery (Gomatos, Krug and Tamm, 1964) that reactions dependent on double stranded RNA are also inhibited by actinomycin. However, there is no conclusive evidence that the resistance of the soluble antigen to digestion with nucleases is due to double or triple strandedness, and its resistance may be attributed to the protein associated with it. Secondly, these assumptions would be in fair agreement with the findings of Wang (1965), for the residual proteins described by him were capable of active amino acid incorporation in vitro. Clark, Chang, Spiegelman and Reichmann (1965) have shown that the 1,200 nucleotides of the RNA of satellite tobacco necrosis virus coded for the 400 amino acid residues of

the virus protein coat. However there does not appear to be sufficient nucleic acid in the soluble antigen to code for the production of the virus protein coat, and therefore it is suggested that this antigen may be responsible for the production of protein associated with the nucleic acid in the internal component of the virus. However, until further evidence is forthcoming this problem must remain unresolved.

The soluble antigen of rabies virus is found only in infected cells, and it would therefore appear that it must either play some role during replication (as suggested above) which as yet is undetermined, or it may merely be a non-functional particle.

As cells infected with virus are in a pathological condition it seems possible that abnormal components such as the soluble antigen may be produced. The three or four rabies soluble antigens (Mead 1962a, b) may be formed at different stages of cell disintergration and they could be considered as abortive virus components brought about by the abnormal conditions of the cell, due to its infection. However, there is no evidence to support this assumption.

Spiegelman and Haruna (1966) have shown that the

DNA dependent RNA polymerase, if presented with a single stranded DNA, synthesizes a DNA-RNA hybrid.
Furthermore, these workers have pointed out that under optimum conditions, as they are known, the prolonged functioning of these enzymes in vitro, can create conditions suitable for the accumulation of abnormal substances.

During the investigation of the soluble antigen of rabies virus the ultraviolet absorption curve of this solution indicated the presence of nucleic acid and protein; it consequently seemed desirable to estimate the relative proportions of each present and to determine the type of nucleic acid present. As only a small volume of dilute antigen solution was available, much time was spent on attempts to adapt the known methods of estimating ribose, deoxyribose and nitrogen to the smallest possible scale of measurement.

The method for the determination of ribose lent itself well to modification. Initially a certain amount of difficulty was encountered where duplicate samples did not agree after the colour had been developed. This lack of agreement was found to be due to an impurity resulting from the method of sealing the tubes.

Reagent prepared with freshly sublimed orcinol gave a more sensitive reaction than the untreated orcinol

and if the concentration of the latter material was increased four fold the sensitivity was further enhanced. An interesting point noted during these investigations was that the blank reading was not increased to any extent by increasing the orcinol concentration. With the introduction of these modifications it was possible to determine as little as 0.025µg ribose/ 0.5ml. with reproducible results.

The method of Burton (1956) for the determination of deoxyribose could not be modified as easily as the orcinol method. The test was performed at concentrations of DNA varying between 1 and 10ug DNA/ml., but the readings obtained at the lower limit of the scale were so low that it was obvious that at the last mentioned concentrations of DNA the test could not be modified to the same extent as that of the orcinol reaction. Initially the calibration curve obtained with DNA standards of different concentration did not pass through the origin, a problem which was also noticed in Fig. 1 of Burtons (1956) article. Two estimations were done on the antigen solution, but as the readings obtained were very near to the lower limits of the test these were accepted as an indication of the presence rather than of the actual concentration of DNA. It is of course possible that the

antigen did not contain DNA and that the colour developed was due to some other substance or substances. Nitrogen determinations were performed according to the method of Jacobs (1964). One of the disadvantages of this method is the high blank readings which could not be reduced by recrystallizing or redistillation of the chemicals used in the preparation of the reagents. The sensitivity of the test was doubled by measuring the absorption of the solutions in a 2 cm. glass cell. Other modifications included increasing the concentration of methyl cellosolve and reducing the concentration of stannous chloride.

The method was very sensitive and had been adapted to an ultramicro scale by Jacobs (1964). Four estimations were done on the antigen solution on successive days, and the results obtained were in good agreement with each other. The antigen solution appeared to contain 11.3µg protein/ml.

In conclusion, it seems that the pentose and nitrogen methods chosen for the estimation of these substances in the antigen solution gave reasonably accurate results. Unfortunately the method of Burton (1956) for deoxyribose estimations was not as easy to adapt to an ultramicro method.

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A B B R E V I A T I O N S.

BS	-	Buffered saline.
cm.	-	Centimetre.
g.	-	Gram.
hr.	-	Hour.
Kcyc.	-	Kilocycles.
l.	-	Litre.
M.	-	Molar.
mm.	-	Millimetre.
mμ.	-	Millimicron.
ml.	-	Millilitre.
min.	-	Minute.
sec.	-	Second.
N.	-	Normal.
rev./min.	-	Revolutions per minute.
S.	-	Svedberg unit of sedimentation.
$^{w}/_{v}$	-	Weight in volume.
$^{v}/_{v}$	-	Volume in volume.
V.	-	Volt.
4^{o}	-	Four degrees centigrade.
mg.	-	Milligram.
μg.	-	Microgram.